OCULAR IMMUNE RESPONSES
clinical and experimental studies

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The investigations described in this thesis have been carried out in the biochemical laboratory (head: Dr. R.M. Broekhuyse) of the Institute of Ophthalmology (director: Prof. Dr. A.F. Deutman) of the Catholic University of Nymegen, The Netherlands.

Aan mijn ouders
Voor Ria, Jolanda en Boukje

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A. INTRODUCTION TO OCULAR IMMUNOLOGY

The anatomical and physiological pecularities of the eye influence the course of some types of ocular inflammation processes. The avascularity of the cornea, lens and vitreous body and the absence of lymphatic drainage make these tissues less accessible to antibodies or immune effector cells. Consequently, they have been considered to be immunologically privileged sites. Especially the absence of microvasculature seems to influence significantly the development of local inflammatory reactions (51, 11). Friedlaender and Dvorak (14) demonstrated that two distinct patterns of delayed-type (cell-mediated) hypersensitivity reactions to the same antigen may occur in skin and cornea, characterised by infiltration of different types of granulocytes. Corneal reactions showed fewer basophils and relatively more neutrophils than the reaction in the cutis.

Another consequence of the relatively isolated situation of cornea, lens and vitreous body as regard to the reticuloendothelial system may be a strong immunogenicity of proteins present in these tissues. Escape of lenticular or corneal proteins during lens extraction or keratoplasty may lead to an autoimmune response manifesting itself as uveitis or keratitis.

Other possible causes of increased immunogenicity of some ocular tissue proteins are irradiation damage, bacterial or viral infection and medicine therapy (antimalarials, steroids) which can lead to tissue damage and/or alterations in protein structures resulting in the formation or release of antigenic components.

Common antigenicity between different intra-ocular tissues and between intra- and extra-ocular tissues is another factor of importance. The involvement of the eye in connective tissue diseases as ankylosing spondylitis and rheumatoid arthritis are supposed to be based on this phenomenon. Increased sensitisation to connective tissue antigens has been demonstrated in rheumatoid arthritis patients (45, 47). Connective tissue antigens are present in all ocular

structures (5, 46, 37, 41, 19). Cross-antigenicity between different intra-ocular tissues might explain the complexity of some ocular inflammation diseases.

B. INTRODUCTION TO GENERAL IMMUNOLOGY

The lymphocytes are shown to be the major cellular elements involved in the immune response. Most of them are circulating through the body in the lymphatics and blood vessels. The bone marrow is the principal source of the lymphoid stem cells in post-natal life. It interacts with the thymus, spleen and lymph nodes in "maintaining the integrity of the lymphoid system" (32). Differences in function and life span seem to be present in the human lymphocyte population, making up a heterogenous collection (16). Systemic studies of clinical immunodeficiency syndromes revealed evidence about the existence of at least two different types of lymphocytes, the thymus dependent (T) lymphocytes and the thymus independent (B) lymphocytes (24, 33, 36) each again consisting of several subpopulations (summarised by Ross (36)). The processing of bone marrow stem cells by thymus and bursa equivalent lymphoid tissue (probably gut-associated lymphoid tissues) to become immunocompetent T- and B-lymphocytes, respectively, is illustrated in Fig. 1.

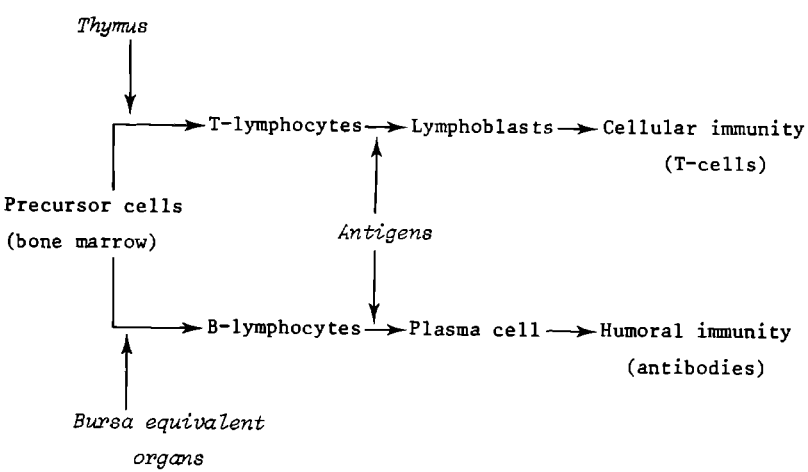

Fig. 1

Antigenic stimulation of B-lymphocytes results in transformation into plasma cells which are responsible for the production of antibodies of various immunoglobulin classes. This type of immune response is known as humoral immunity and can passively be transfered to normal recipients by means of serum. The T-lymphocytes, on the other hand, are only indirectly involved in the synthesis of antibodies by cooperation with the plasma cells. Antigenic stimulation of the T-lymphocytes leads to transformation into activated lymphoblasts which are involved in the so-called cellular immune reactions (26), which can passively be transfered to normal recipients by means of lymphocytes and not by serum.

Although the T-lymphocytes do not secrete antibodies, they have the capacity to release other biologically active molecules, known as lymphokines (12). The classification and characterisation of some lymphokines has been reviewed (7, 49). While of some lymphokines the activity is known, the biological function of most of these mediators still remain obscure.

C. I. DEMONSTRATION OF HUMORAL IMMUNE REACTIONS

Methods for the detection of the immediate-type hypersensitivity or humoral immune reactions have been described by many authors. The majority of the techniques is based on the direct interaction between antibodies and the appropriate antigens or on the consequences of this interaction. By allowing antibody and antigen to diffuse in gels a precipitation reaction may be formed which may become visible after staining. Immunodiffusion techniques are based on this principle (50). One of the consequences of the interaction between antibody and antigen may be the modification of the antibody molecule structure resulting in the activation of a set of biological phenomena which may be detected (7). This principle is used in techniques as the hemagglutination test (21) and the complement fixation test (35).

II. DEMONSTRATION OF CELLULAR IMMUNE REACTIONS

Skin test- In contrast to immediate-type hypersensitivity, the research on delayed-type hypersensitivity or cellular immunity has been hampered until the last decennia by the lack of sophisticated in-vitro methods. Cellular immunity may be detected by intracutaneous

injection of the appropriate antigen. If, because of a previous contact, the individual has been sensitised to the injected antigen, an erythematous indurative lesion develops after several hours reaching a maximum at 24-48 hours. This technique has been used as an application in tuberculosis research and is still the most practical method of assessing the presence or absence of cellular immunity in patients. A number of restrictions, for example the inability to employ tissue-associated antigens, drugs or infectious agents and the fact that the reaction gives only a picture of a local process, made it necessary to search for new in-vitro techniques (42, 34).

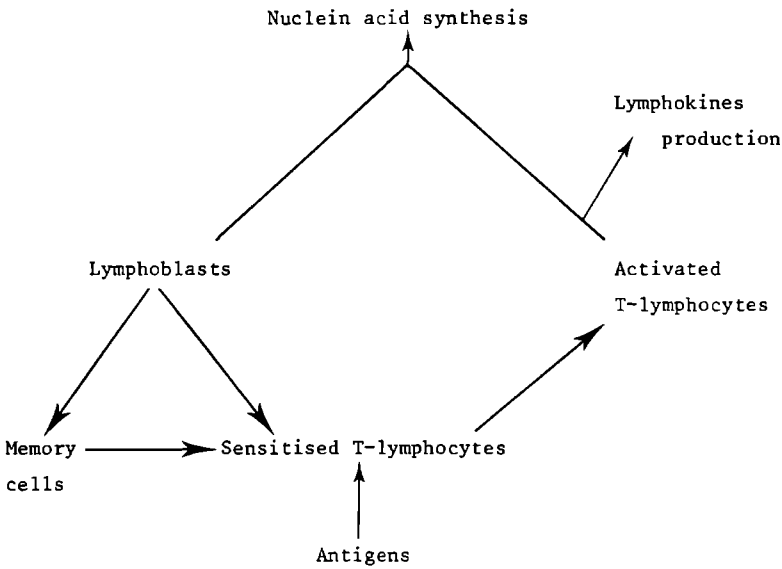

Fig. 2

In Fig. 2, illustrating the lymphocyte activation cycle (49), it is shown that the production of lymphokines precedes the transformation of the activated T-lymphocytes into lymphoblasts. The presence of a delayed-type hypersensitivity state can therefore be detected by lymphocyte transformation (blastogenesis) or by the production of lymphokines.

Blastogenesis- Blastogenesis is the process of the transformation of small lymphocytes into large pyroninophilic immunoblasts

characterised by a high cytoplasm/nucleus ratio. This process has been first described by Nowell (30) after incubation of lymphocytes with an extract of beans (phaseolus vulgaris) called phytohemagglutinin (PHA). Subsequently, numerous other products were discovered having the capacity to trigger the transformation process (26).

The process of lymphocyte transformation eventually results in DNA synthesis and cell division. The intensity of the transformation process of the whole cell population may be measured visually by morphological evaluation or by the incorporation of radioactive purine or pyrimidine. The latter method is assumed to be more sensitive and objective, since there appears to be no stringent correlation between DNA synthesis and morphologic blastogenesis (43).

The technique described in our studies is based upon the incorporation of tritiated thymidine into the DNA of the activated lymphocyte and should better be called lymphocyte stimulation. Fleer et al. (13) demonstrated a good correlation between the results of the skin test and those of the lymphocyte stimulation test using PPD (purified protein derivate) as an antigen. Immunisation of rabbits with bovine corneal epithelium antigens also revealed appropriate accordance between skin reactivity and lymphocyte stimulation (4). Moreover, studies of Blomgren (2) and Weksler and Kuntz (52) clearly showed that purified human B-lymphocytes do not respond to PPD or PHA whereas isolated human T-lymphocytes do, although mixing of both populations revealed a synergy between both cell types.

Sell (38, 39) described blast transformation of rabbit lymphocytes after incubation with antisera to rabbit immunoglobulins, which are present on the membrane of B-lymphocytes, suggesting a possible stimulation of these cells. However, the eventuality that the blood T-lymphocytes of rabbits display surface immunoglobulins may not be ruled out (40).

In conclusion, although the stimulation of human lymphocytes by mitogens or antigens is now extensively employed as a general assessment of cell-mediated immune function "it would almost certainly be erroneous to decide that B-lymphocytes may not similarly respond to antigens" (17). This made it necessary to use complementary methods to estimate cellular immune reactions in human beings as well.

Lymphokine production- The production of biologically active macromolecules or lymphokines by lymphocytes after contact with anti-

gens or mitogens (Fig. 2) might be another valuable tool for the detection of cellular immunity. The macrophage migration inhibition factor (MIF), causing inhibition of migration of guinea pig macrophages (3) was one of the first lymphokines used for the detection of delayed-type hypersensitivity reactions (15, 9, 10). The macrophage migration inhibition method of George and Vaughan (15) has been modified by Bendixen and Søborg (1) using peripheral blood leukocytes. This technique has been called the leukocyte migration inhibition technique and is based on the production of a leukocyte migration inhibition factor (LIF) after contact between lymphocytes and antigens or mitogens. A large variety of inhibition methods has been described, partially based on different technical procedures. The agarose droplet technique as used in our studies has been developed according to the method as described by Harrington and Stastny (20) for guinea pig macrophages and by McCoy et al. (27, 28) for human leukocytes.

The utility of the leukocyte migration inhibition assay for the detection of delayed-type hypersensitivity has been demonstrated by several investigators. Hoffman et al. (22, 23) clearly showed the leukocyte migration inhibition test to be an excellent in-vitro correlate of skin reactivity and macrophage migration inhibition in guinea pigs and human beings. A good correlation between the leukocyte migration inhibition test and the skin test was demonstrated by others as well (6, 25, 36). Destruction of T-cells with anti-T-cell antiserum eliminated LIF activity suggesting the involvement of T-lymphocytes in the production of this lymphokine (18). This suggestion was supported by the abnormal LIF activity present in children with diseases concerning the T-cell system (18). On the contrary, Fleer and colleagues (13) were not able to demonstrate a clear and reproducable correlation between leukocyte migration inhibition and skin reaction.

Although B-lymphocytes (53, 8) and even a number of non-lymphoid tissues can be induced to synthetise migration inhibitory products (44, 48, 31) it is still accepted that the synthesis of lymphokines results from antigenic stimulation of sensitised T-lymphocytes (8).

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CHAPTER 1

LYMPHOCYTE STIMULATION AND LEUKOCYTE MIGRATION INHIBITION: TECHNICAL DESCRIPTION.

Lymphocyte stimulation – Human blood was taken from the cubital vein and anticoagulated with heparin (Thromboliquin, Organon, Oss). Rabbits were bled from the marginal ear vein or from the heart. The blood was diluted with an equal volume of Tris buffered Eagle's Minimum Essential Medium, containing spinner salts and L-glutamine, supplemented with 100 units penicillin and 100 µg streptomycin per ml, pH 7,2-7,4 (MEM, Gibco Bio-Cult Ltd., New York) and layered over half the volume of a Ficoll-Isopaque mixture consisting of 9.556 g Ficoll 400 (Pharmacia Fine Chemical AB, Uppsala, Sweden) mixed with 20 ml Isopaque (440 mg I/ml, Nyegaard & Co. AS, Oslo). The density of the Ficoll-Isopaque mixture had been adjusted to 1.077 g/cm^3 (20°C) with distilled water and sterilised by heat. The blood-Ficoll-Isopaque gradient was centrifuged at 1000 x g for 20 minutes at 20°C. The interphase containing the lymphocytes was collected and washed 2 times with MEM by centrifugation at 400 x g for 10 minutes. A sample of the mononuclear cells was stained with Türk's solution (Merck, Darmstadt) and counted in a counting chamber. Human lymphocytes were diluted to a concentration of 0.3 x 10^6 cells per ml with MEM containing 20% A-rh positive heat-inactivated human serum. Rabbit lymphocytes were diluted to a concentration of 1 x 10^6 lymphocytes per ml with MEM containing 15% homologous heat-inactivated rabbit serum. One ml of these suspensions was pipetted into sterile tubes (NUNC, grad nr. 1090, with caps nr. 1095) and antigen or mitogen solution was added in appropriate amounts. Lymphocytes cultured without antigen or mitogen served as controls. As demonstrated in Table I, the amount of lyophilised ocular tissue antigens needed for maximal lymphocyte stimulation depended on the

Table I. *Concentration of various ocular antigens for maximal stimulation of human and rabbit lymphocytes.*

Ocular antigens	Human lymphocytes*	Rabbit lymphocytes**
Soluble corneal epithelium (Bo)	1000 µg	500 µg
Insoluble corneal epithelium (Bo)		100 µg***
Soluble corneal stroma (Bo)	500 µg	500 µg
Corneal structural glycoprotein (Bo)		50 µg
Soluble cornea (Hu)	1000 µg	500 µg
Soluble sclera (Bo)		1000 µg
Soluble choroid (Bo)		500 µg
Soluble choroid (Hu)	500 µg	
Soluble retina (Hu)	200-500 µg	500 µg
Soluble retina (Bo)	200 µg	500 µg
Soluble retina (Pi)	200 µg	500 µg
Soluble retina (Mo)	200 µg	500 µg
Insoluble retina (Hu)	50 µg	
Rod outer segments (Bo)	50 µg	
Uveal pigment granules (Bo)	10 µg	
Soluble lens (Bo)	1000 µg	500 µg
Soluble lens (Hu)	1000 µg	
Soluble lens (Ra)	1000 µg	500 µg
Alpha crystallin (Bo)	50 µg	50 µg
Alpha crystallin (Ra)	50 µg	50 µg
Soluble iris (Bo)		500 µg

Values represent amount of lyophilised material (µg) per ml.
Bo, bovine; Hu, human; Pi,pig; Mo, monkey; Ra, rabbit.
Culture period : *6-7 days, ** 5 days, ***4 days.

type of eye tissue. Human lymphocytes and rabbit lymphocytes needed different culture periods for maximal stimulation with the various antigen fractions. The mitogen reactivity was determined by incubating human lymphocytes during 3 days with 5 µg and rabbit lymphocytes during 2 days with 50 µg of phytohemagglutinin (PHA-P; Difco). Twenty-four hours before harvesting, 0.5 µCi ^{3}H-thymidine (6-^{3}H-thymidine, spec. activity 20-30 Ci/mmol; The Radiochemical Centre, Amersham) was added. The cells were harvested by filtration under reduced pressure through Millipore glass-fiber filters (Millipore, AP 2002500). The filters containing human lymphocytes were incubated in 0.5 ml NCS (Amsterdam, Tissue Solubilizer, 0.6N solution, Searle) diluted 1 to 3 with toluene based scintillation fluid (Packard Becker, MI 92), containing 100 mg dimethyl-POPOP and 5 g PPO per litre, for 30 minutes at 20°C. After addition of 9.5 ml toluene based scintillation fluid, containing 1 ml/1 glacial acetic acid, the activity was measured in a liquid scintillation counter. Filters containing rabbit lymphocytes were dried for 18 hours at 45°C or 30 minutes at 100°C and incubated in 6 ml toluene based scintillation fluid. Because the efficiency of the used system was consistently 40%, the activity was represented by counts per minute (cpm). The degree of lymphocyte stimulation was expressed as the stimulation index (S.I.) calculated according to the following formula:

$$S.I. = \frac{\text{mean cpm measured in lymphocytes cultured with antigen}}{\text{mean cpm measured in lymphocytes cultured without antigen}}$$

Leukocyte migration - Under sterile conditions four and half ml blood was collected from patients or healthy persons and anti-coagulated with heparin. The blood was mixed with 1,5 ml sterile 5% Dextran 200 (MW 200 000) in saline and incubated at 37°C for 40 minutes. The buffy coat cells were collected and centrifuged at 1000 x g for 5 minutes at 20°C. The cells were washed 5 times with tissue culture medium 199 (GIBCO,Bio-Cult Ltd., New York, with Earle's Salts and L-glutamine) supplemented with 100 units penicillin and 100 µg streptomycin per ml and 10% heat-in-

activated sterile horse serum (GIBCO) (Complete Medium 199). The leukocytes were incubated in 2 ml Complete Medium 199. A sample was stained with Türk's solution and counted in a counting chamber. The cells were resuspended in 0,2% agarose (Indubiose A45, L'industrie Biologique Francaise, SA-Gennevilliers) in Complete Medium 199 in a final concentration of 2.2 x 10^8 cells per ml. Droplets of 4 µl of this suspension were placed in migration chambers (Leukocyte Migration Plate, nr. 308, Sterilin, Teddington, Middlesex). The droplets were allowed tot solidify for about 5 minutes at 20°C and incubated in Complete Medium 199 with or without antigens. The type of antigen and the antigen concentrations which were used are shown in Table II. After covering the chambers with glass slides, the cells were allowed to migrate at 37°C for 18-20 hours. The area of migration was measured with an ocular micrometer (magnification 7 x; Bausch and Lomb) as was the area of the agarose droplets. The migration index (M.I.) was calculated as follows:

M.I. = mean migration area of cells in presence of antigen-mean area of drops/mean migration area of cells in absence of antigen-mean area of drops x 100%

Table II. *Concentration of various ocular antigens for maximal human leukocyte migration inhibition.*

Ocular antigens	Antigen concentration
Soluble lens (Hu)	300 µg
Soluble retina (Hu)	400 µg
Soluble choroid (Hu)	400 µg
Insoluble retina (Hu)	100 µg
Rod outer segments (Bo)	100 µg
Rhodopsin (Bo)	10 µg
Uveal pigment granules (Bo)	20 µg

Values represent amount of lyophilised material (µg) per ml.
Hu-human; Bo-bovine.

The development of iritis (38), uveitis (22) and retinal oedema (8) in association with extra-ocular manifestations often encountered after repeated injections of horse serum in human beings suggests that the eye participates as an integral part of the body in systemic hypersensitivity reactions. Inflammatory diseases of the eye seem to be related with a number of rheumatic diseases such as juvenile rheumatoid arthritis, ankylosing spondylitis (Bechterew)(24, 35) and Reiter's syndrome (17). However, in 1940 Dean et al. (13) described 6 cases suffering from interstitial keratitis caused by a specific sensitivity to food, without the presence of any systemic allergic manifestation, suggesting the possibility of a local ocular sensitisation. The production of chorioretinitis and uveitis in animals after systemic injection of autologous retinal extracts (21) or after intraperitoneal implantation of autologous eyes (40) support the possibility of an autosensitisation resulting in local ocular inflammation.

Circulating immune complexes may play a role in the pathogenesis of some local ocular inflammations. The presence of immune complexes consisting of antigen, antibody and complement in the aqueous humor and retina and an alteration in ocular vascularity could be demonstrated in rabbits after intravenous challenge of immunised animals or by infusion of antigen-antibody complexes (25, 23, 14, 6) suggesting the involvement of type III hypersensitivity.

In this type of hypersensitivity, antigen-antibody complexes are present which may give rise to activation of the complement system and subsequent formation of anaphylatoxins. The latter are responsible for the release of histamine from platelets which results in vascular permeability changes. In addition the formation of chemotactic factors and activation of the Hageman factor cause accumulation of polymorphonuclear leukocytes and thrombotic occlusions, respectively.

Complement components have been detected in the aqueous humor of human eyes (10). High concentrations of immunoglobulin-G are present predominantly, in cornea and choroid (1, 2). Lower concentrations were found in other parts of the human eye including the aqueous humor (2, 10, 44, 30). These findings certainly indicate the possibility of the involvement of type III hypersensitivity in patients suffering from ocular diseases as described in the beginning of this chapter. Indeed Dernouchamps et al. (14) found immune complexes in the aqueous humor of 5 out of 13 patients suffering from endogenous uveitis.

According to Maumenee (28) inflammation of the uvea rarely is a primary event but it is usually the result of an immunological activity directed to another part of the eye. This theory is partially supported by the experiments of Silverstein (34) which clearly demonstrated that cells responsible for the incipience of uveitis had developed extra-ocularly and may persist within the uvea for months, making the eye behave like a lymph node. However, antibodies to uveal pigment granules or soluble uveal fraction have been demonstrated in patients suffering from injury of the uveal tract (43), endogenous uveitis (19, 26) and lens-induced uveitis (26). These results reinforce the hypothesis that some kinds of uveitis may have a "direct" immunopathological etiology. The development of uveitis in guinea pigs after sensitisation with allogenetic uveal material (3, 4) or limbal tissue (12) supports this theory. Moreover, these animal studies revealed that in addition to the earlier mentioned antigenicity of the uveal pigment granules, the soluble part of the pigmented uvea contains at least two distinct antigens, one representing characteristics of the pigmented uvea, the other of the albino (3). Sensitisation of rabbits with uvea isolated from albino or pigmented rabbit eyes incorporated in adjuvant, however, did not result in ocular inflammation despite the fact that anti-uvea antibodies and skin reactivity to uveal antigens were present (37).

The development of experimental allergic uveitis after passive transfer of hyperimmune lymph node cells (5) does suggest the involvement of cellular immune reactions in this type of uveal disease. The implantation of specifically sensitised autologous lymph node tissue in the anterior chamber of rabbit eyes followed

by intracorneal injection with the appropriate antigen resulted in a marked iritis with aqueous humor flare and fibrinous exudate. This supports the view that, within the eye, immunologically competent cells may respond to local sensitisation, resulting in clinical uveitis (16). The involvement of cellular immune reactions has been demonstrated by the presence of macrophage chemotactic factors in the aqueous humor of rabbits during experimental immunogenic uveitis (31) and by the injection of lymphokines in the vitreous of guinea pigs (9). Chemotactic factors for rabbit polymorphonuclear cells could be detected in the aqueous humor of patients suffering from Behçet's syndrome (32, 33). The participation of cell-mediated immunity in this type of endogenous uveitis could be confirmed through incubating leukocytes of these patients with autologous vitreous or aqueous humor (29). Indeed immune suppresive treatment of patients with Behçet's syndrome by azathioprine and cortison improved the course of this disease (15). Cellular immune activity seems to be absent in other forms of endogenous uveitis as determined by lymphocyte transformation (18) or leukocyte migration inhibition (36). The stimulation of isolated lymphocytes of patients suffering from sympathetic ophthalmia or Vogt-Koyanagi-Harada syndrome by uveal pigment (20) suggests that cell-mediated immune activity to uvea may play a role in these diseases. This work tends to be confirmed by the results found by Wong et al. (42) and by Marak et al. (27). The observation that patients suffering from Vogt-Koyanagi-Harada syndrome manifest an alteration in the percentage of thymus derived peripheral blood lymphocytes (11) would support the concept that an immune mechanism may be important in the development or progression of this disease. No gross alteration in the immune status in patients with sympathetic ophthalmia could be observed (7).

In mice, the induction of experimental uveitis was prevented when T-lymphocytes, sensitised to choriomeningitis virus, were destroyed by anti-θ-antibody and complement before they were passively transfered, although the simultaneously transfered B-lymphocytes did produce antibodies. Thus, in mice lymphocytic choriomeningitis virus induced ocular pathology is based upon a mechanism of cellular immunity (39).

In conclusion, despite the fact that contradictory results have been found concerning the participation of either humoral or cellular immunity in some forms of uveitis, no doubt remains on the existence of the immunological nature of some types of uveitis. Ultrastructural analysis of human iris biopsies in 17 cases with uveitis with different etiology revealed that all cells involved in immunological reactions were present (41). However, presence of immunocompetent cells does not necessary indicate an immune reaction directed to uveal tissue. These cells (small and medium sized lymphocytes, plasma cells and various types of macrophages) may be involved in the neutralisation of locally released or produced antigens.

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CHAPTER 2

Experimental Uveitis in Isolated Humoral and Cellular Immunity

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Summary Passive transfer of homologous immune serum in rabbits followed by intravitreous injection with the corresponding antigen resulted in an uveal inflammation which resembled the Arthus-type reaction Clinically and histologically, the reaction was maximal 24 h after antigen injection The histologically observed cell infiltration consisted predominantly of polymorphonuclear leukocytes

Passive transfer of sensitized homologous thymocytes followed by intravitreous injection with the corresponding antigen resulted in an uveal inflammation which resembled the delayed-type hypersensitivity reaction Tissue infiltration of polymorphonuclear cells as well as mononuclear cells occurred predominantly during the first day following antigen injection An exudate containing almost exclusively eosinophils was present in the aqueous humor and/or vitreous body of most of these rabbits During the second day an increase in the ratio mononuclear cells/polymorphonuclear cells could be observed

Introduction

The pathogenesis of various forms of uveitis is not yet well understood Antibodies to human uveal antigens have been demonstrated in patients suffering from endogenous uveitis (Hallett et al , 1962, Luntz, 1968), indicating the participation of B-lymphocytes in this disease The successful application of corticosteroids to patients suffering from uveitis suggests an involvement of the cellular immune system Conflicting reports have been published about the role of cellular immunity in uveitis, based merely on the use of different techniques and antigens (Strandgaard and Braendstrup 1971, Feinberg et al , 1972, Freedman and Smit, 1974, Henley et al 1975) Moreover, apart from a true immunological reaction to uveal antigens, uveitis could be a manifestation of

an immunological reaction to antigens from other parts of the eye (Rahi and Garner, 1976)

It has been shown that immunized rabbits develop a severe uveitis after intravitreous injection with the corresponding antigen Transfer of immune serum followed by intravitreous antigen injection resulted in an inflammation resembling the Arthus phenomenon (Waksman and Bullington 1956, Silverstein and Zimmerman 1959) The transfer model has been used in the present study to investigate and to compare the role of both the humoral and the cellular immune system in endogenous uveitis by passive transfer of immune serum and sensitized thymocytes, respectively, followed by intravitreous injection of the corresponding antigen

Materials and Methods

Animals New Zealand rabbits weighing about 5 lb of either sex were used

Antigens The soluble fraction of bovine corneal epithelium (EpSo) was isolated as described previously (Brinkman et al 1978) and dissolved in sterile saline to a concentration of 10 mg dry weight per ml The solution was sterilized by ultrasonic treatment

Immunization The rabbits were injected subcutaneously at multiple sites on the back with 15 mg EpSo solubilized in 1 ml phosphate buffered saline (PBS) and emulsified with 1 ml Freund's complete adjuvant (FCA) If boosting was performed 15 mg EpSo solubilized in 1 ml PBS was injected subcutaneously 4 weeks later

Sera and Thymocytes Collection The sensitized rabbits were exsanguinated The blood was centrifuged and suitable sera were pooled All sera were stored at −20° C Immediately after exsanguination the thymus was removed and incubated in sterile Medium 199 (Gibco) Fat and other tissues were trimmed off and the thymi still incubated in Medium 199 were cut into small pieces The thymocytes were isolated by teasing these pieces with sterile forceps Tissue aggregates were removed by filtration through sterile gauzes The cells were washed twice with Medium 199 and counted Viability was determined by trypan blue exclusion and amounted to almost 100%

Sera and thymocytes of unsensitized rabbits were isolated in the same way

Passive Transfer Immune serum was transferred by intravenous injection into the marginal ear vein Each rabbit (S rabbit) received 15 ml serum on days 1 3 5 8 and 10 The last serum injection was just before intravitreous injection Controls received normal serum The sensitized thymocytes were resuspended in sterile Medium 199 and injected intravenously in the marginal ear vein of a second group of rabbits Each rabbit (T rabbit) received $3\,6\ 4\,6 \times 10^9$ thymocytes in a volume of 6 10 ml 18 h before the intravitreous injection Controls received thymocytes of nonimmunized rabbits

Intravitreous Injection The rabbits were anesthesized with Hypnorm (0 5 ml/kg) to facilitate injection Ten microliters saline containing 100 µg EpSo was injected into the vitreous of the right eye The left eye which received 10 µl sterile saline served as control The eyes were observed 6 24 and 48 h later with the slit lamp The severity of the inflammation included aqueous flare (Tyndall) iris hyperemia proteinaceous exudate and fibrin strands cells in the anterior chamber and posterior synechias Each sign was scored separately on a scale of 0 to 4+ Posterior synechias were scored as 0 (absence) or 1+ (presence) The summation of the total scores is considered to be representative for the severity of the inflammation (Ashford and Lamble 1974)

Histological Examination The animals were killed by Nembutal 24 or 48 h after the intravitreous injections and the eyes enucleated for fixation in glutaraldehyde The globes were progressively

dehydrated in alcohol and embedded in paraffin Sections of 8 µm were cut and stained with methyl green-pyronine and hematoxylin eosin for histological evaluation Cell infiltration was graded on a scale of 0 to 4+ depending on the density of cell infiltration

Skin Tests Skin tests and intravitreous injections were carried out simultaneously Fifty microliters saline containing 100 µg EpSo was injected intradermally The reactions were recorded after 6 24 and 48 h Erythema and indurations were graded separately on a scale of 0 to 4+ Necrosis was scored as 0 (absence) and 1+ (presence) The degree of the skin reaction is represented by the summation of the scores

Lymphocyte Culture Ten ml blood was withdrawn from donor rabbits just before killing and from the recipient rabbits 24 h after intravitreous injection for lymphocyte culture and antibody titer determination Isolation culturing labeling and harvesting of the rabbit lymphocytes were carried out as described in a previous paper (Brinkman et al 1978) The degree of stimulation is reported as the stimulation index (SI) which is expressed as the ratio of the ^{3}H-thymidine incorporation into the DNA of lymphocytes cultured in the presence of EpSo to that in the absence of EpSo An SI $\geqq$2 was considered to be positive

Antibody Titer Determination of the antibody titer was performed by the hemagglutination test (Herbert 1973) using 1 ml 2% (v v) tanned sheep erythrocytes incubated with 1 mg EpSo

Results

Serum Transfer Three pools of anti-EpSo antiserum having an antibody titer of 3,000, 15,000 and 50 000, respectively and normal serum were transferred to separate groups of rabbits as described Twenty-four hours after intravitreous injection of 100 µg EpSo into the right eyes of the recipient rabbits, a clinically detectable inflammatory reaction was present in 11 (91%) of the 12 passively immunized rabbits (Table 1) Occasionally it could be detected even after 6 h The most pronounced reaction was iris hyperemia occurring in almost every animal Sometimes fibrinous deposits could be observed at the pupil margin Aqueous flare was present in all rabbits which received the immune serum The inflammation resolved rapidly during the following 24 h Rabbits which received normal serum showed occasional aqueous flare but never iris hyperemia The control left eyes of all animals remained normal after saline injection No striking differences could be detected in the degree of the clinically observed inflammation following transfer of anti-EpSo antisera with different antibody titers as demonstrated in Table 1

Unlike the reaction in the eyes, the skin reaction was at its maximum 6 h after intradermal EpSo injection For the skin test this indicates an immediate-type immune reaction Control rabbits never developed a positive skin reaction (Table 1) Only one of the control rabbits presented in Table 1 showed a weak positive lymphocyte stimulation with EpSo, while cellular immunity was not detectable in the other rabbits

Histological sections of the right eyes showed a degree of cell infiltration which was maximal 24 h after intravitreous antigen injection (Table 1) The infiltration consisted predominantly of polymorphonuclear (PMN) cells present especially in the limbus region (Fig 1) and iris root, and sometimes around the optic nerve Plasma cells and blast-type cells were scarcely present Forty-eight hours after the intravitreous injection the total cell infiltration decreased as

Table 1 Ocular reaction evoked by intravitreous antigen injection following anti EpSo antiserum and normal serum transfer

Rabbit number	Antibody titer	LST[a]	Skin reaction			Clinical severity (OD)[b]			Histological severity[b] (OD)		
			6 h	24 h	48 h	6 h	24 h	48 h	24 h	48 h	exudate[d]
8	1 000	N D		N D		0	7+	5+		2+	+
9	900	N D		N D		0	7+	2+		2	+
10	1 280	0 8	5+	3+	2−	0	4+	0		0	0
11	2 560	0 7	5+	2+	1+	3+	11+	0		+	+
12	1 280	0 7	5+	2−	1+	0	9−	0		2+	+
13	1 280	1 0	3+	1+	1+	15+	18+	3+		+	+
14	1 280	0 6	4+	2+	2+	1−	8+	0		0	+
15	1 280	0 6	3−	2+	1+	0	0	0		2−	+
16	5 000	0 6	6+	3+		0	8+		3+		+
17	5 000	0 9	4+	2+		0	15+		4+		+
18	10 000	0 8	7+	2		0	6+		+		+
19	10 000	1 0	7+	2+		0	10+		+		+
1 3	0	0 8±0 2	0	0	0	0	+	−		0	0
4 7	0	1 0±0 7	0	0		0	+		0		0

Rabbits 8 9 received anti EpSo antiserum with antibody titer of 3 000
Rabbits 10 15 received anti EpSo antiserum with antibody titer of 15 000
Rabbits 16 19 received anti EpSo antiserum with antibody titer of 50 000
Rabbits 1 7 received normal serum

[a] Lymphocyte stimulation test determined 24 h after intravitreous injection values represent SI

[b] Density of cell infiltration range 0 4+
Score levels are explained in Materials and Methods

[d] Exudate in vitreous and or anterior chamber 0 (absence) + (presence)

compared to eyes enucleated 24 h following intravitreous antigen injection In almost all eyes exudates could be detected in the vitreous and/or anterior chamber containing some MN (mononuclear) and PMN cells in many cases The left eyes were histologically without a sign of uveitis The right eyes of two control animals showed a few PMN cells in the limbus region The eyes of the other control rabbits remained normal

Transfer of Thymocytes The thymocytes were isolated from two groups of donor rabbits The first group of rabbits was injected once with EpSo with FCA Three weeks after immunization peripheral blood lymphocytes were isolated for culturing with EpSo The average SI amounted to $3\,5\pm1\,3$ ($n=5$) as compared to $1\,0\pm0\,2$ ($n=5$) for non-immunized rabbits The animals were killed and their thymocytes isolated pooled and transferred to recipient rabbits (numbers 37 40) Intravitreous injection and skin tests with EpSo were performed 18 h later Clinically no reaction could be registered after 6 24 and 48 h (Table 2) Skin tests were also negative Histological evaluation of

Fig. 1. Infiltration of PMN cells in Limbus region of rabbit eye after immune serum transfer followed by intravitreous antigen injection. The eye was enucleated 24 h later (Hematoxylin and eosin, ×250)

the right eyes showed no infiltration, neither after 24 h, nor after 48 h following intravitreous antigen injection.

The donor rabbits of the second group were injected with EpSo with FCA and boosted with EpSo without FCA 4 weeks later. Two weeks after boosting their peripheral blood lymphocytes were cultured with EpSo. The average SI amounted to 24.5 ± 21.0 ($n=14$). The animals were killed and their thymocytes were isolated from the thymi, pooled, and transferred to 17 recipient rabbits (numbers, 20–36). Skin tests and intravitreous injections with EpSo were given 18 h later. Eight rabbits (numbers, 20–27) were killed after 24 h, and nine (numbers 28–36) after 48 h. Clinically, no reaction could be observed after 6 h. After 24 h the right eyes of 14 (82%) of 17 animals showed the clinical

Table 2 Ocular reaction evoked by intravitreous antigen injection following transfer of sensitized and unsensitized thymocytes

Rabbit number	Clinical severity (OD)[a]			Histological severity (OD)[b]		
	6 h	24 h	48 h	24 h	48 h	exudate
20	0	7−		0		0
21	0	12−		+		+
22	0	5+		2+		+
23	0	9+		3+		+
24	0	8−		3+		+
25	0	3+		0		0
26	0	4+		0		0
27	0	5+		+		0
28	0	4+	1+		+	+
29	0	2+	3+		0	0
30	0	3+	2+		3+	+
31	0	4+	1+		0	0
32	0	6+	2+		0	0
33	0	0	0		0	0
34	0	9+	6−		3+	+
35	0	0	3+		0	0
36	0	0	0		0	0
37	0	0	0		0	0
38	0	0	0		0	0
39	0	0	0		0	0
40	0	0	0		0	0
41–44	0	0	0	0	0	0

Rabbits 20–36 received thymocytes of animals with an average SI of the peripheral lymphocytes of 24.5 ± 21.0

Rabbits 37–40 received thymocytes of animals with an average SI of the peripheral lymphocytes of 3.5 ± 1.3

Rabbits 41–44 received thymocytes of unsensitized animals with an average SI of the peripheral lymphocytes of 1.0 ± 0.2. Both eyes were injected with 100 µg EpSo. OS was removed 24 h and OD 48 h after the intravitreous injection

[a] Score levels are explained in Materials and Methods

[b] Density of cell infiltration: range 0–4+

[c] Exudate in vitreous and/or anterior chamber: 0 (absence), + (presence)

signs of uveitis. The reaction slowly vanished during the following 24 h (Table 2). The reactions consisted especially of aqueous flare, iris hyperemia, and cells in the anterior chamber (Fig. 2). Usually, the degree of reaction was less severe than that of animals which received immune serum followed by intravitreous EpSo injection. Skin tests and anti-EpSo antibody titers of the recipient rabbits were consistently negative. The left eyes of the rabbits showed occasional aqueous flare after saline injection. The control rabbits (numbers 41–44) which received unsensitized thymocytes were intravitreally injected with 100 µg EpSo

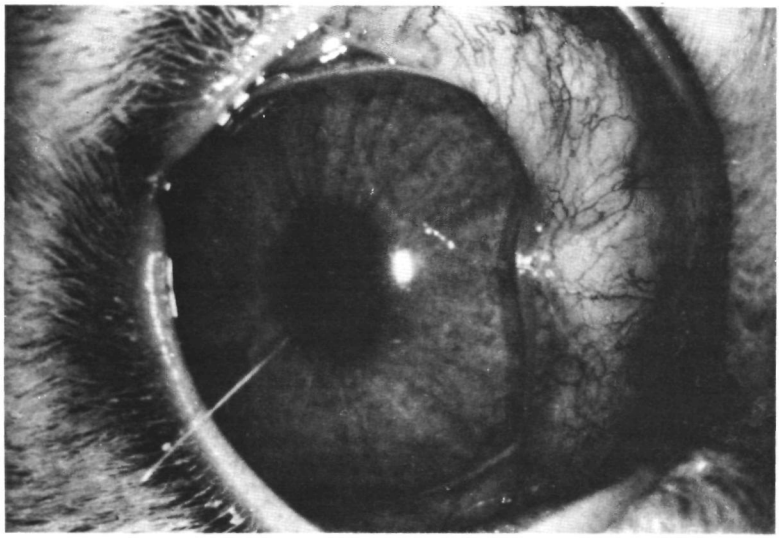

Fig. 2. Iris hyperemia and exudate in aqueous humor of rabbit eye after passive transfer of sensitized thymocytes followed by intravitreous antigen injection. Reaction was recorded 24 h later

in both eyes. Twenty-four hours later the left eyes were removed for histological evaluation. The right eyes were removed 48 h following intravitreous injection (Table 2). Clinically, no reaction could be observed 24 and 48 h following intravitreous injection (Table 2).

Histological examination of the eyes of the eight rabbits (numbers, 20–27) killed after 24 h and which were intravitreally injected with 100 μg EpSo following transfer of sensitized thymocytes revealed a tissue cell infiltration, consisting of PMN cells and less MN cells, in five (62%) cases. An exudate containing almost exclusively eosinophils was present in the aqueous humor and/or vitreous body in four of these rabbits (Fig. 3). Three (33%) of the nine rabbits (numbers 28–36) killed after 48 h following intravitreous EpSo injection showed a cellular infiltration, consisting of MN cells and PMN cells in almost equal ratios and occasionally blast-type cells and plasma cells. The inflammatory cells were present especially in the limbus region (Fig. 4) and iris root, and sometimes in the ciliary body. The control left eyes showed a few cells in the limbus region in two cases (12%). This infiltration was always less severe than in the fellow right eyes. None of the eyes of the rabbits which received unsensitized thymocytes followed by intravitreous EpSo injection in both eyes reacted positively, neither after 24 h nor after 48 h.

Fig. 3. Exudate consisting predominantly of eosinophils present in the aqueous humor of rabbit after transfer of sensitized thymocytes followed by intravitreous antigen injection. The eye was enucleated 24 h later (Hematoxylin and eosin, × 125)

Discussion

To what extent cellular and humoral immunity participate in the induction of uveitis is still a matter of uncertainty. Conflicting results have been published about the presence of cellular immunity in patients suffering from certain forms of uveitis (Strandgaard and Braendstrup, 1971; Feinberg et al., 1972, Freedman and Smit, 1974; Henley et al., 1975). The contribution of different immunological mechanisms in producing nongranulomatous endophthalmitis has been studied earlier, by varying the method of sensitization and by passive transfer of immune serum (Waksman and Bullington, 1956; Silverstein and Zimmerman, 1959), followed by intravitreous antigen injection. An isolated response of the cellular part of the immune system can hardly be accomplished by active immunization.

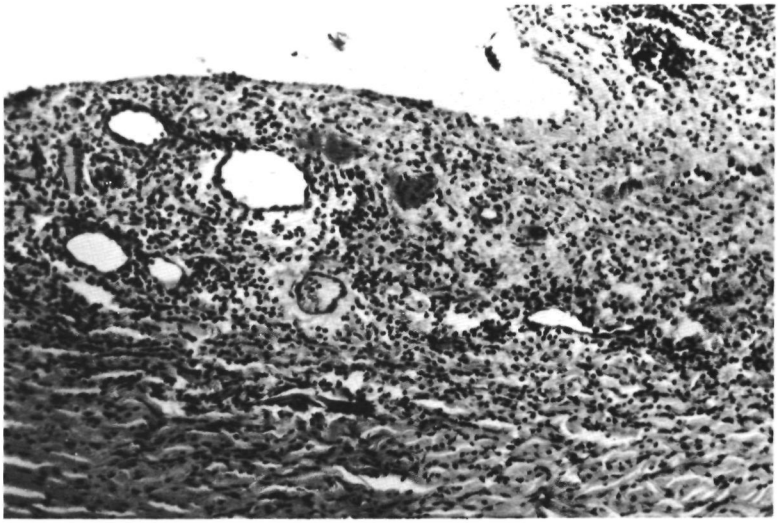

Fig. 4. Infiltration of MN and PMN cells in limbus region of rabbit eye after passive transfer of sensitized thymocytes followed by intravitreous antigen injection. The eye was enucleated 48 h later (Hematoxylin and eosin, × 125)

Experimental allergic uveitis could be produced in healthy guinea pigs by passive transfer of cells isolated from guinea pigs suffering from chronic uveitis (Aronson and McMaster, 1971). By using the cells from spleen and lymph nodes which contain B-lymphocytes as well as T-lymphocytes, no clear separation between cellular and humoral immunity was obtained. The lymphocytes isolated from the thymus of the rabbit have been shown to be exclusively T-lymphocytes (Sabolivic et al., 1977), and consequently may be suitable for the study of the role of cellular immunity in eliciting uveitis, assuming that they have been sensitized. Utilizing passive transfer of immune sera and sensitized thymocytes, followed by intravitreous injection of the antigen (EpSo), we have demonstrated in the present study that humoral as well as cellular immunity may play an individual role in the onset of uveitis.

Earlier studies in our laboratory have shown the strong antigenic nature of EpSo (Brinkman et al., 1978). Intravitreous injection of this antigen after transferring immune serum resulted in an inflammation which could sometimes be detected even after 6 h. Clinically and histologically, the reactions were most pronounced after 24 h following intravitreous antigen injection and declined sharply thereafter. The slow disappearance of the antibodies from the blood of the recipient rabbits (as was estimated) could hardly account for this decline. The failure of the in vitro stimulation of the peripheral blood lymphocytes of the S-rabbits by the corresponding antigen indicates the absence of cellular immunity. The rapid onset of the inflammation in some cases and the intense

hyperemia of the iris suggest that an Arthus-type reaction was involved, which is in agreement with the results of other investigators (Waksman and Bullington, 1956 Silverstein and Zimmerman, 1959) Antigen-antibody complexes precipitate within the walls of the blood vessels Subsequently, complement is activated, and chemotactic factors and anaphylatoxin are released Histamine, secondarily to anaphylatoxin, causes an acute increase in vascular permeability, resulting in severe iris hyperemia and exudates in the vitreous and anterior chamber As a consequence of the liberation of the chemotactic components, leukocytes, predominantly PMN cells, accumulate in the limbus region and iris 24 h after intravitreous antigen injection Skin tests of the S-rabbits showed also the typical Arthus-type reaction, reaching a maximum about 6 h after antigen injection and then resolving The delay of the onset of the Arthus reaction in the eye, as compared to the skin, might be explained by the slow release of antigen out of the vitreous, caused by a long diffusion distance and by the blood-aqueous barrier (Fernando, 1960, Hasty et al , 1971) The observed variability in the onset of the raction may be due to a variability in the localization of the intravitreous antigen depot

Transfer of sensitized thymocytes followed by intravitreous antigen injection led to a different type of inflammation process Firstly, clinical symptoms were noticed not earlier than 24 h following the intravitreous antigen injection Secondly, the inflammatory process was less severe than that in serum-treated animals and included especially aqueous flare and iris hyperemia Forty-eight hours after the intravitreous antigen injection, most rabbits still showed a clinically well-detectable syndrome suggesting a delayed-type hypersensitivity reaction This supposition is supported by the absence of anti-EpSo antibodies in the recipient rabbits The correlation between the stimulation of the peripheral blood lymphocytes of the donor rabbits, and the ability of their thymocytes in transferring the immune activity also indicates the relation between cellular immunity and the onset of uveitis

An infiltration of both PMN cells and MN cells could be detected in the limbus and iris of positively reacting T-rabbits, 24 and 48 h following intravitreous antigen injection The PMN cells (especially eosinophils) present in the observed exudates suggest the release of lymphokines during the G1 phase of activated T-lymphocytes (Valdimarsson, 1976) One of these mediators is chemotactic for eosinophils (Cohen 1976) The difference in severity and period of occurence of uveitis in S- and T-rabbits following intravitreous antigen injection might be explained by an interaction between immunologically competend cells of the recipient rabbits and the donor thymocytes A host versus graft reaction is an inevitable rule in the case of histocompatibility difference between donor and recipient animals and regularly leads to damage of most of the transferred thymocytes, which may also explain the negative skin tests demonstrated by the T-rabbits However, the reactions within the eye can be observed at a lower sensitivity threshold due to the transparency of some ocular media

The results of the present investigation suggest that both humoral and cellular immunity can play a role in the pathogenesis of some forms of uveitis Particu-

larly those types of uveitis which resemble the animal model used in this investigation, like nongranulomatous iridocyclitis, phacogenic uveitis, and possibly uveitis arising after cryotherapy of patients who have suffered from retinal detachment. Uveal inflammatory processes as a consequence of bacterial infections might also be candidates. It has been found that endotoxin molecules, present at the cell membranes of most of the gram negative bacteria, are most efficient in stimulating the complement system (Gewurz, 1971), of which components have been demonstrated in the aqueous humor of human beings (Chandler et al., 1974). The resulting inflammation might resemble the uveitis described in this report after immune serum transfer followed by intravitreous antigen injection.

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During the last 15 years particular attention has been paid to the significance and commitment of the retina in several diseases of the eye. The possible role of retinal antigens in the production of uveitis was first suspected by Wacker and Lipton (24). Guinea pigs injected with homologous retinal tissue emulsified with Freund's complete adjuvant developed uveitis, characterised by an infiltration of predominantly mononuclear cells in choroid and ciliary body. The lesions were always bilateral and occurred primarily in the uvea and only secondarily in the retina (24). Comparable results were found with monkeys after immunisation with homologous retina although the difference between the severity of the uveitis and the retinopathy was less evident (19). In these animals, retinal periphlebitis was preceded or accompanied by the development of a broad perivascular halo. In rabbits, involvement of the retina in the destruction process following immunisation with homologous retinal extracts (17) or autologous retinal extracts (7) could also be demonstrated. The clinical picture was characterised by chorioretinitic patches of varying sizes in all parts of the fundus. The lack of destruction processes in the anterior segment and in the central nervous system (15), suggested the specificity of the inflammation process. Histologic examination revealed a cellular infiltration of choroid and retina, swelling of pigment epithelial cells and destruction of the retinal photoreceptor layer (16). Scanning electron microscope studies on auto-immune uveo-retinitis in guinea pigs evoked by immunisation with autologous retinal extracts revealed in the anterior chamber the presence of numerous lymphocytes which appeared to immigrate through the ciliary epithelium. Progression of the disease led to retinal detachment and massive infiltration of polymorphonuclear leukocytes in the subretinal space. The rods in the photoreceptor layer had disappeared (14). The study of Wacker (20) clearly demonstrated the inflammation pro-

cesses produced in guinea pigs either with homologous uvea or retina to be antigenically and pathologically different diseases indicating the specificity of the retinal antigens.

With respect to the retinal involvement in varying species of animals the differences in clinical and histological reactions appear partially to be attributable to differences in retinal vascular supply. It has been shown that in rats and rabbits the characteristics of the ocular inflammation responses following intravitreal injection of antigen were similar in the anterior segments but different in the posterior segments. In rats, the inflammation of the posterior segment secondarily to intravitreal injection of antigen involved primarily the retinal vessels, due to the extensive retinal vasculature (1). Wacker and Kalsow (23) also clearly demonstrated the influence of intra-ocular vascularisation. Following immunisation with homologous retinal antigens, guinea pigs, classified as paucangiotic, primarily developed choroiditis and only secondarily retinitis. On the contrary, in the rat retinitis was detected as an initial manifestation of the inflammation process whereas the choroid was only mildly involved. Thus in rats the retinal circulation appears to be the source of retinal infiltration (23).

Various attempts have been made to fractionate retinal tissues in order to identify the antigenicity of the different components. Wacker and Lipton (25) analysed the antigenic properties of the soluble and particulate parts of retinas of guinea pigs. While in these animals antibodies to both preparations were formed upon injection, the production of allergic uveitis could be demonstrated with the soluble portion of the retinal preparation only. Since this fraction elicited a high response of the cellular immune system in the animals these results argue against the participation of humoral antibodies in the pathogenesis of the disease. However, De Kozak et al. (2) described the successful passive transfer of uveitis with anti-total retina antiserum and the presence of immune complexes in the inflamed ocular tissues of guinea pigs. On the other hand, peripheral lymphocytes of guinea pigs immunised with homologous retinal extract, displayed cytotoxicity against their retinal and uveal target cells after in vitro incubation, thus suggesting the involvement of a cellular immune response in this desease (5).

A more detailed analysis of the soluble antigenic fraction of the retinas of guinea pigs resulted in the identification of a protein with highly pathogenic properties (S-antigen) localised in the photoreceptor layer. Immunisation of guinea pigs with as little as 5 µg of this allologous retinal specific soluble protein produced 100% incidence of uveitis (21). The molecular weight of this antigen amounted to 55000 dalton and in addition to the guinea pig it has been demonstrated also in retinas of bovine (22), rabbit and man (4). Speculations have been made about the nature of this S-antigen. Similarities in amino-acid composition were found with a retinal-binding protein present in the outer segments of rods (22). The presence of this S-antigen in the photoreceptor layer of the retina might clarify the antigenicity of this layer as it was demonstrated by Rahi (12) and confirmed by Hempel et al. (8) and Wong et al. (26). These authors demonstrated the possibility to evoke intra-ocular inflammation in both rabbits and monkeys following systemic immunisation with homologous rod outer segments. Clinically, the monkeys showed fundus lesions, opacity of the vitreous body, oedema of the nerve head and extensive arterial and venous perivasculitis (26). Histological studies revealed remarkable changes of the outer segments of the photoreceptor layer leading to complete destruction of the outer segments. An intense inflammation process was observed in the retina, subretinal space and choroid. The appearance of mononuclear cells in the anterior uvea, the trabecular meshwork and the vitreous body suggests the involvement of the cellular part of the immune system. From these studies it seems reliable to suggest the accumulating (infiltrating) lymphocytes to be sensitised to rod outer segments and to exert the decisive role in the pathogenesis of clinical chorioretinitis (10). Furthermore, in the leukocyte migration inhibition test Healey et al. (9) demonstrated that soluble retinal antigens produced a significant migration inhibition of leukocytes of patients with chorioretinitis suggesting the participation of these antigens in this disease.

It should be stressed that anti-rod outer segments antibodies are more likely to have protective functions rather than pathogenic functions. Rabbits, immunised with homologous rod outer segments and showing the highest antibody titers did not develop chorioretinitic lesions in contrary to animals with low antibody titers (18).

Similar studies were performed with guinea pigs. Uveo-retinitis induced after systemic immunisation with homologous retinal extracts could be suppressed by multiple series of injections of this antigenic preparation before or after the onset of the eye disease. The repeated injections produced an increase in the antibody titer and a depression of the cellular immune system (3).

In addition to the S-antigen the antigenic properties of the rod outer segments might be based on the presence of significant amounts of rhodopsin. Studies of a possible influence of this lipoglycoprotein on the induction of some forms of eye diseases are rather scarce. It has been mentioned that an immune response developing to homologous rhodopsin may result in complete blindness in monkeys because of destructive events in the outer segments of the retina (27). A comparable phenomenon was described by Faure et al. (6) in rabbits after systemic injections of partially purified rhodopsin.

In conclusion, no doubt exists about the strong antigenicity of the retina as compared with other intra-ocular tissues. Besides a strong antigenic soluble protein predominantly located in the photoreceptor layer of the retina, it seems reasonable to suggest that rhodopsin itself is antigenic. The latter might explain the observed antigenicity of the retinal pigment epithelium (11, 13) which is known to play a role in the breakdown of rod outer segments and which consequently might contain sufficient high concentrations of remnants of these antigenic retinal fragments.

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CHAPTER 3

CELL-MEDIATED IMMUNITY AFTER RETINAL DETACHMENT AS DETERMINED BY LYMPHOCYTE STIMULATION

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Delayed hypersensitivity to an antigen can be measured in vitro by incorporating tritiated thymidine into the DNA of the sensitized lymphocytes that have been cultured in the presence of the antigen Cell-mediated immunity to antigens, including those from eye tissues, can also be ascertained by estimating the production of biologically active mediators, or lymphokines, released by sensitized lymphocytes upon contact with a specific antigen [1,2] The leukocyte migration inhibition test, based on the production of a leukocyte migration inhibition factor, has been done on cells from patients suffering from several eye diseases, including retinal detachment However, conflicting results have been published regarding the validity of the leukocyte migration inhibition test as an indicator of cell-mediated immunity [3-7] The lymphocyte stimulation test seems more sensitive and objective in detecting cell-mediated immunity [7]

By investigating lymphocyte stimulation on incubation with human and bovine soluble retinal antigens, and serum antibodies directed at these antigens, we determined whether patients suffering from retinal detachment exhibited cellular immunity

MATERIAL AND METHODS

We studied 29 patients suffering from retinal detachment, and nine healthy persons None of the patients received corticosteroid therapy, which could inhibit lymphocyte stimulation [8]

Retinas were dissected from human donor eyes within 48 hours' storage at 4° C and from fresh bovine eyes All further procedures were carried out at 0 to 4° C unless otherwise indicated The retinas were pooled and homogenized in a Potter-Elvehjem tube in excess 0 01M ammonium acetate at pH 7 4 The soluble antigen fraction was isolated by centrifuging for 60 minutes at 15,000 × g The supernatant was lyophilized and the product (ReSo) stored at −20° C in small batches until used The antigen solutions were sterilized by sonication

Protein content was determined by using bovine serum albumin as a standard [9]

A sample of heparinized blood was used for plasma preparation, and the remainder was diluted with an equal volume of Eagle's Minimal Essential Medium The mononuclear cells were isolated from the diluted blood by centrifugation [10] Lymphocytes, 3×10^5, were suspended in 1 ml of Eagle's Minimal Essential Medium supplemented with 100 units of penicillin and 0 1 mg of streptomycin per milliliter and 20% heat-inactivated A-rh positive serum Cultures of lymphocytes were incubated in triplicate with 0 005 mg of phytohemagglutinin-P for 72 hours, and with 0 5 mg of human or 0 5 mg of bovine ReSo for six to seven days The same batch of antigen was used for all tests Control cultures contained neither mitogen nor antigen Twenty-four hours before harvesting, 0 5 μCi of tritiated thymidine (specific activity, 29 4 Curi/mM) was added Cells were harvested by filtration under reduced pres-

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sure through Millipore glass fiber filters The filters were incubated in 0 5 ml of solubilizer diluted in a ratio of 1 3 with toluene-based scintillation fluid contain ing 100 mg of di-methyl-POPOP and 5 g of PPO per liter, for 30 minutes at 20° C After addition of 9 5 ml of scintillation fluid, containing 1 ml of glacial acetic acid per liter, the activity was counted in a liquid scintillation counter The degree of stimulation was expressed as the stim ulation index (S I) S I is the ratio of tritiated thymidine incorporation (counts per minute) into lymphocytes cultured in the presence of antigen to those cultured in the absence of antigen Correction was made for background activity An S I ≥ 2 with human ReSo and an S I ≥ 3 with bovine ReSo were considered to be positive

The serum antibody titer was estimated by the hemagglutination test [11] One milliliter of 2% weight by volume tanned sheep erythrocytes was incubated with 1 mg of ReSo from human or bovine source A serum antibody titer of 10 or more was considered to be positive

Results

Preliminary studies indicated that the highest tritiated thymidine incorporation into the human lymphocytes of patients with retinal detachment occurred during the culture period of six to seven days with 0 5 mg of dry weight retinal antigen (about 0 25 mg of protein) extracted from human or bovine tissue

These conditions were used to investigate the degree of sensitization of other patients suffering from retinal detachment (Table 1) Of the 29 patients, 12 (41%) showed a positive lymphocyte stimulation on incubation with human or bovine ReSo, whereas nine healthy controls did not (Table 2) The difference between the number of patients who showed a positive lymphocyte stimulation and the number of healthy controls who did not was statistically significant ($P< 05$, Fisher's one-sided exact test) Only four of 15 patients with detachment of less than four weeks' duration showed a positive lymphocyte stimulation test, which was statistically insignificant when compared to the group of healthy controls ($P> 10$) Eight (57%) of 14 patients who suffered from retinal detachment for more than about four weeks had a positive lymphocyte stimulation This was statistically significant when compared to the group of healthy controls ($P< 01$) There was no correlation between the degree of the lymphocyte stimulation after phytohemagglutin-P incubation and that after ReSo incubation

A positive serum antibody titer to human retinal antigens was detected in 60% of all patients (Table 1) Of 15 patients suffering from retinal detachment for less than about one month, 11 (73%) had a positive serum antibody titer to human retinal antigen as compared to six (46%) of the 13 patients with prolonged retinal detachment Healthy controls did not show a positive serum antibody titer to human or bovine retinal antigen (Table 2) The difference between the number of healthy controls and the number of patients suffering from retinal detachment for less or more than one month and showing a positive serum antibody titer was statistically significant ($P< 0001$ and $P< 05$, respectively)

Fifteen patients suffered not only from retinal detachment, but also from cataract Ten (67%) of these patients showed a positive serum antibody titer to human retinal antigen (Table 1), which was significantly more than three (30%) of the ten patients with no accompanying ocular disorders showing a positive serum antibody titer to human retinal antigen ($P< 10$, Fisher's two-sided exact test)

Only five of the 12 patients with posi-

TABLE I

LYMPHOCYTE STIMULATION AND SERUM ANTIBODY TITER OF PATIENTS SUFFERING FROM RETINAL DETACHMENT

Case No., Sex, Age (yrs)	Accompanying Ocular Disorders	Duration of Retinal Detachment	PHAP*	Stimulation Index ReSo (Hu)†	Stimulation Index ReSo (Bo)‡	Serum Antibody Titer ReSo (Hu)	Serum Antibody Titer ReSo (Bo)
1, M, 56	Cataract, diabetic retinopathy	<1 wk	29 7	0 7	1 2	0	0
2, M, 33	Cataract	<1 wk	51 5	1 3	1 7	0	0
3, F, 59	Cataract	<1 wk	29 8	1 2	1 2	200	200
4, M, 58	Cataract	1 wk	19 6	0 7	0 6	200	100
5, F, 49	Myopia	1 wk	27 6	3 9	3 5	50	10
6, F, 56	None	<2 wks	23 1	4 6	N D §	0	0
7, F, 29	None	<2 wks	28 9	0 6	0 9	100	200
8, F, 61	Cataract	<2 wks	14 6	0 7	1 7	200	100
9, M, 19	Cataract, myopia	<2 wks	20 4	0 9	1 4	100	100
10, F, 76	Macular degeneration	<2 wks	154 5	5 6	47 1	100	100
11 M, 57	Cataract	<2 wks	33 9	1 5	1 2	100	50
12, M, 65	None	2 wks	61 7	2 7	7 6	0	0
13, F, 51	None	2 wks	40 1	1 2	1 6	50	0
14, M, 44	Congenital cataract	<3 wks	112 4	1 5	1 1	400	400
15, F, 54	Cataract	<3 wks	147 6	1 4	0 7	800	200
16, M, 64	None	4 wks	37 6	2 9	N D	0	0
17, M, 73	None	4 wks	7 2	0 5	0 2	0	0
18, F, 41	None	4 wks	55 5	0 7	0 7	0	50
19, F, 60	Trauma	<5 wks	30 3	1 7	2 0	200	200
20 F, 83	Cataract	5 wks	137 8	2 6	6 5	100	100
21 F, 29	None	5 wks	63 0	1 0	1 4	0	10
22, F, 59	None	5 wks	22 1	0 8	0 9	100	10
23, M, 72	Trauma	<6 wks	21 9	1 2	3 1	100	50
24, F, 63	Cataract	> 2 mos	19 3	0 8	1 5	10	10
25, F, 67	Cataract	2 5 mos	13 0	4 0	N D	0	N D
26 F, 75	Cataract	3 mos	31 0	0 7	3 1	N D	0
27 M, 58	Cataract	9 mos	45 4	2 9	3 6	0	0
28, M, 14	None	13 mos	31 8	3 0	N D	0	50
29, M, 35	Cararact, myopia	36 mos	136 9	27 6	57 0	50	0

*PHAP designates phytohemagglutinin-P
†S I ≧ 2 is considered to be positive Hu designates human
‡S I ≧ 3 is considered to be positive Bo designates bovine
§N D designates not determined

TABLE 2

LYMPHOCYTE STIMULATION AND SERUM ANTIBODY TITER OF CONTROLS

Case No	Sex Age (yrs)	Stimulation Index PHA‡	Stimulation Index ReSo(Hu)*	Stimulation Index ReSo(Bo)†	Serum Antibody Titer ReSo(Hu)	Serum Antibody Titer ReSo(Bo)
1	M 34	41 4	1 9	2 1	0	0
2	M 30	15 9	1 9	2 5	0	0
3	M 26	46 7	0 9	1 2	0	0
4	F 25	46 1	1 7	2 1	0	0
5	F 23	22 2	1 5	2 6	0	0
6	F 19	35 2	1 0	2 2	0	0
7	F 32	34 9	1 6	2 0	0	0
8	M 27	20 0	1 1	1 4	0	0
9	M 24	92 3	0 8	1 7	0	0
	Average	39 4 ± 22 8§	1 4 ± 0 4	2 0 ± 0 5	0	0

*S I ≥ 2 is considered to be positive Hu designates human
†S I ≥ 3 is considered to be positive Bo designates bovine
‡Stimulation Index ± S D

tive lymphocyte stimulation also showed a positive serum antibody titer to human ReSo (Table 1) The results of the lymphocyte stimulation test and the hemagglutination test appear in Table 3

DISCUSSION

Experimental uveitis induced by injecting animals with several retinal extracts confirms the antigenic nature of the retina [12 17] Our report reveals that patients suffering from retinal detachment may exhibit lymphocyte stimulation in vitro with human and bovine retinal extracts The lack of sufficient human donor eyes for retinal antigen extraction forced us to test the influence of bovine retinal antigen on human lymphocytes The results in Table 1 show a close relationship in lymphocyte stimulation after incubation with both human and bovine retinal antigen This same relationship is true for antibody determination This possible common antigenicity is now being more closely investigated In our study, lymphocyte stimulation after incubation with bovine retinal antigen was, in most cases, higher than after incubation with human retinal antigen, which might be explained on the basis of difference in chemical structure, as well as the histoincompatibility between man and calf However, a stimulation index of three or more in the case of the bovine antigen was never found with controls (Table 2) and was therefore used as a criterion for a positive reaction The correlation between the duration of the detachment and the presence of sensitized lymphocytes strongly suggests that an immunological process is involved Cellular immunity to retinal antigen is present in only four (26%) of the 15 patients suffering from retinal detachment for less than about one month, which proves that immunity is a secondary process and becomes detectable

TABLE 3

RELATIONSHIP BETWEEN IMMUNITY AND THE DURATION OF RETINAL DETACHMENT

Immunity to Retinal Antigens	Duration of Retinal Detachment* ≤4 wks	Duration of Retinal Detachment* >4 wks
Positive stimulation index	4 15 26)	8 14 (57)
Positive antibody titer	11 15 73)	6 13 (46)

*Number of total patients percentages are indicated in parentheses

about four weeks after detachment These results corroborate the study of Henley, Okas, and Leopold[18] who used the leukocyte migration inhibition test as the in vitro test for detecting cellular immunity

After cryotherapy, which is accompanied by pigment fallout, [19] uveitis may occur in patients suffering from retinal detachment because of the presence of sensitized lymphocytes and because the retina shares at least one antigen with the other parts of the eye [20 21] Other explanations for the onset of uveitis are the formation of dead tissue and the consequent release of tissue proteases

More than half of the patients suffered not only from retinal detachment, but also from cataracts (Table 1), and 70% of the cataract patients demonstrated a serum antibody titer to lenticular antigens of more than 50 (data not shown) Because common antigenicity exists between lenticular and retinal antigens,[20] the high number of patients having positive serum antibody titer to retinal antigens (Tables 1 and 3) may be explained by the presence of antibodies to lenticular antigens This correlation between cataract and the presence of antibodies to "retinal" (lens) antigens is supported by the fact that the group of patients with prolonged retinal detachment contained the smallest number of patients suffering from cataract (40%) and the smallest number of patients having a positive serum antibody titer to retinal antigen (46%) In the group of patients with a retinal detachment present for less than one month, 60% suffered from cataract and 73% had a positive serum antibody titer

Fleer and associates[7] demonstrated that the lymphocyte stimulation test is accurate in detecting cell-mediated immunity In our study no correlation between a positive serum antibody titer to retinal antigens and the presence of lymphocytes sensitized to retinal antigens could be detected We also demonstrated that the presence of sensitized lymphocytes depends on the duration of the detachment We therefore believe the sensitization that may arise after retinal detachment and that is measured by the lymphocyte stimulation test is cell-mediated

Summary

We examined 29 patients suffering from retinal detachment for the presence of lymphocytes sensitized to retinal antigens by performing the lymphocyte stimulation test Twelve patients (41%) showed a positive lymphocyte stimulation upon incubation with soluble retinal antigens, whereas nine healthy controls did not The occurrence of sensitized lymphocytes depended on the duration of the detachment and could be detected in eight (57%) of the 15 patients suffering from the detachment for more than about four weeks

A positive serum antibody titer to human retinal antigen was found especially in patients suffering from retinal detachment for less than about four weeks We detected no correlation between the presence of sensitized lymphocytes and a positive antibody titer The controls did not have serum antibodies directed to retinal antigens The results suggest cellular immunity after retinal detachment In our patients, bovine soluble retinal antigens stimulated their lymphocytes to a similar or higher extent than human soluble retinal antigens, which suggests the possibility of replacement of human by bovine retinal antigens

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CHAPTER 4

IMMUNE REACTIVITY TO DIFFERENT RETINAL ANTIGENS IN PATIENTS SUFFERING FROM RETINITIS PIGMENTOSA

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SUMMARY

The immune status of patients suffering from different types of retinitis pigmentosa has been investigated. The lymphocytes of these patients could be stimulated by incubation with human soluble retinal antigens as well as with bovine rod outer segments. The results suggested the involvement of the cellular immune system in retinitis pigmentosa. The leukocyte migration inhibition test also pointed in that way, especially if bovine rhodopsin was used as the antigen. The complement fixation test suggested the presence of a non-specific weak antibody activity in the blood of retinitis pigmentosa patients as well as of controls. This activity seemed predominantly to be directed to the insoluble fraction of human retinas.

On the basis of the findings we conclude that patients suffering from retinitis pigmentosa may become sensitized to retinal antigens, especially to those localized in the rod outer segments. This sensitization concerns the cell-mediated immune system and seems not to be correlated with a special type of the disease.

INTRODUCTION

Retinitis pigmentosa, characterized by specific ophthalmoscopic and electrophysiological abnormalities, night blindness and constriction of the visual field (28), is one of the most frequently encountered inherited eye diseases. Although the pathogenesis is still obscure, studies performed on laboratory animals, suffering from retinal degeneration showing some resemblance with the human retinal di-

sease (11), suggested a defect in the phagocytic mechanism of the retinal pigment epithelium (7), resulting in an accumulation of rod outer segments (12, 11). The antigenicity of the retina especially of the rod outer segments has been demonstrated earlier in-vivo (20, 27, 10, 29, 9, 15, 26). The accumulated rod outer segments or other retinal antigens might evoke an immune response which could amplify or accelerate the disease. Previous studies of cell-mediated immunity on patients suffering from retinitis pigmentosa using the macrophage migration inhibition test yielded positive results with human retinal antigens (24).

The objectives of the present communication were to investigate the presence of a humoral or cellular immune response in retinitis pigmentosa and to compare the antigenic properties of different retinal fractions.

MATERIALS AND METHODS

Subjects- The study comprised 23 patients (average age: 26 years; range 13-48 years) suffering from clinically well documented retinitis pigmentosa. The patients received no steroid therapy at the moment of testing. The 24th. subject did not suffer from retinitis pigmentosa but was carrier of the autosomal recessive form of this disease. This subject was considered as control. Thirteen healthy volunteers (average age: 27 years; range 21-46 years) and 22 patients (average age: 41 years; range 14-80 years) suffering from various eye disorders (including: optic atrofy (3), corneal diseases (12), APMPPE (1), myopy (1), retrobulbar neuritis (1), retinal pigment epithelium alterations (1), central cone disturbance (1), low vision, normal ERG (2)), served as controls.

Eleven ml blood was withdrawn from a cubital vein of each person and anticoagulated with heparin. One ml blood was used for plasma preparation, 5 ml blood for lymphocyte isolation and 5 ml blood for leukocyte isolation.

Antigen preparation- All procedures concerning antigen preparation were conducted at 0-4^{o} C. The soluble fraction of human retinas (Hu-ReSo) was isolated by homogenization and centrifugation as previously described (1). Protein content on dry weight basis amounted to 50% according to the method of Lowry et al. (18) using bovine

serum albumin as standard. The 450,000g.min sediment of human retinas was washed several times with 0.01 M ammonium acetate buffer (pH 7.4) until the supernatant remained protein free. The sediment was lyophilized and the product (Hu-ReIn) was stored at -20° C until used. During dissection of the human retinas the photoreceptor layers appeared to stick to the corpus vitreous. Fluorescence microscopy with rabbit anti-bovine rod outer segments antiserum did not reveal the presence of photoreceptor fragments in Hu-ReIn. Complete bovine retinas showed bright fluorescence with this antiserum. The protein content of the dried sediment amounted to 50% according to the method of Lees and Paxman (17) using sodium dodecylsulphate as solubilizer. Rod outer segments (Bo-ROS) and rhodopsin (Bo-Rho) were isolated from fresh bovine retinas by sucrose gradient centrifugation (5) and were a gift of Dr. W.J. de Grip (Nijmegen). The A_{278}/A_{500} ratio of the rhodopsin preparation amounted to 1.6. In this preparation, phospholipids and proteins other than rhodopsin could not be detected (2). Bo-Rho was solubilized (2mg/ml) in pyperazine-N, 'N-bis [2-ethane sulfonic acid] containing 20 mmol nonylglucose. The purification of bovine uveal pigment granules (Bo-UP) has been described (3).

Lymphocyte stimulation- Five ml heparinized blood was diluted with 5 ml Eagle's Minimum Essential Medium (Gibco). Isolation, culturing, labeling and harvesting of the lymphocytes have been described (1). Lymphocyte stimulation was carried out by incubating 0.3 x 10^6 lymphocytes, in triplicate, with 200 µg Hu-ReSo, 50 µg Hu-ReIn, 50 µg Bo-ROS and 10 µg Bo-UP for 6-7 days at 37° C. These values represented optimal quantities (µg dry weight) for the particular batches of antigens. The antigens were sterilized by ultrasonic treatment. Viability of the lymphocytes was checked by incubation with 5 µg phytohemagglutinin-P (Difco) for 3 days. The degree of lymphocyte stimulation is given as the stimulation index (SI) which is expressed at the ratio of ^{3}H-thymidine incorporation into lymphocytes cultured in the presence of antigen to ^{3}H-thymidine incorporation into lymphocytes cultured in the absence of antigen. An SI $\geq$ 2 with Hu-ReSo or Hu-ReIn and an SI $\geq$ 3 with Bo-ROS or Bo-UP were considered to represent a positive reaction (1).

Leukocyte migration inhibition- A modification of the agarose droplet technique as developed by Harrington and Stastny (8) has been

used. Five ml heparinized blood was mixed with 1.5 ml 5% Dextran 200 (M.W. 200,000) in saline. After incubation for 40 min at 37° C, the buffy coat cells were collected and washed 6 times with Hepes buffered tissue culture medium (Medium 199; Gibco). The leukocytes were incubated in 0.2% agarose (Indubiose A37, l'Industrie Biologique Française, Gennevilliers, France) in Medium 199, containing 10% horse serum and antibiotics, in a final concentration of 2.2×10^8 cells per ml. Drops of 4 µl were placed in migration chambers (Sterilin, leukocyte migration plate, No. 308). After 5 min, the chambers were filled with 0.4 ml Medium 199, containing 10% horse serum and antibiotics, without or with antigens in the following concentrations (based on dry material): Hu-ReSo, 400 µg; Hu-ReIn, 100 µg; Bo-ROS, 100 µg; Bo-Rho, 10 µg; and Bo-UP, 20 µg per ml. Each antigen was tested in quadruplicate. The chambers were covered and incubated for 18-20 hr at 37° C. The diameters of the cell migration areas and of the agarose drops were measured with an ocular micrometer. The migration index (MI) was calculated according to the formula:

$$MI = \frac{\text{mean migration area of cells in presence of antigen-mean area of drops}}{\text{mean migration area of cells in absence of antigen-mean area of drops}} \times 100\%$$

An MI ≤ 75% with either antigen fraction was considered to represent a positive reaction.

Antibody titer- Plasma (0.1 ml) of retinitis pigmentosa patients and controls was heat-inactivated (30 min, 56° C) and diluted with veronal buffered saline, pH 7.3, to several concentrations (up to 1:640). Bo-Rho (1 µg), Bo-ROS (1 µg), Bo-UP (1 µg), Hu-ReSo (25 µg) and Hu-ReIn (100 µg) were added in 0.1 ml veronal buffered saline. To this mixture 0.1 ml 1:15 diluted guinea pig complement (Flow Laboratories) was added. After 16 hr at 4° C, 0.2 ml 2% (v/v) amboceptor (diluted 1:40,000; Flow Laboratories) sensitized sheep erythrocytes was added. Lysis was measured by spectrophotometry at 415 nm after incubation for 1 hr at 37° C. A lysis of ≤ 75% was indicative for the presence of neutralizing antibodies in the plasma.

The clinical data of the investigated subjects suffering from retinitis pigmentosa are compiled in Table I. The ERG response was non-recordable in the majority of the patients and subnormal in the others. In this table subject No. 24 was carrier of the disease.

The number of isolated lymphocytes or leukocytes from some retinitis pigmentosa patients and controls was not sufficient to test all antigens. This is reflected in the varying numbers of tested subjects (see Figs. 1 and 2).

Lymphocyte stimulation- The stimulation indices of retinitis pigmentosa patients and control subjects after incubation with different ocular antigens are given in Fig. 1.

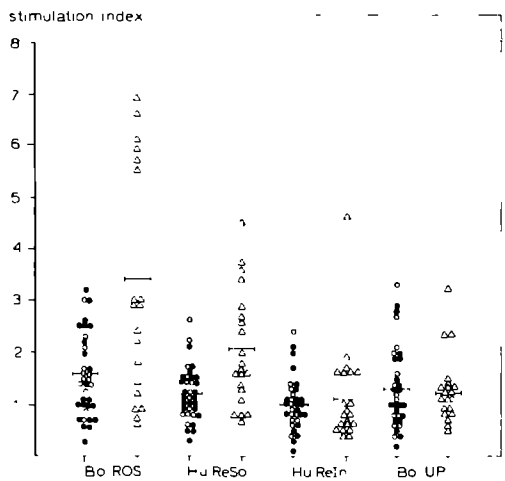

Fig. 1. *Stimulation of lymphocytes of patients suffering from retinitis pigmentosa* (Δ) *and of controls in response to different ocular antigens. The group of the controls consisted of healthy subjects* (o) *and patients suffering from eye diseases other than retinitis pigmentosa* (●). *Horizontal bars indicate the mean stimulation indices.*

Average stimulation indices are indicated by horizontal bars. Statistical analysis by comparing individual SI's (Mann and Whitney U-test) revealed significant differences in lymphocyte activation between retinitis pigmentosa patients and controls after incubation of their lymphocytes with bovine rod outer segments ($P = 0.008$) and soluble human retinal antigens ($P = 0.002$). No differences between both groups of subjects could be detected after incubation of their lymphocytes with insoluble human retinal antigens ($P = 0.74$) or bovine uveal pigment granules ($P = 0.09$). Considering an SI = 2 with

Table I *Clinical data of patients*

Patient No.,	Sex,	Age	Type of retinitis pigmentosa	ERG response	Accompanying disorders
1	M	36	S	non-recordable	cataract, nystagmus
2	M	39	S	non-recordable	
3	M	46	S	non-recordable	cataract
4	M	13	AD	subnormal	
5	F	15	AD	subnormal	
6	F	44	AD	non-recordable	
7	M	30	AR	non-recordable	consanguinity
8	M	48	AD	non-recordable	cells in vitreous, cataract
9	M	22	AD	non-recordable	cells in vitreous
10	F	22	AD	subnormal	
11	F	26	AD	subnormal	
12	F	36	AD	subnormal	leakage of retinal capillaries
13	M	30	AD	subnormal	peripheral retinal vasculopathy
14	M	15	XL	non-recordable	
15	M	37	S	non-recordable	
16	F	25	S	non-recordable	nystagmus
17	F	17	AR	non-recordable	consanguinity
18	F	21	AR	non-recordable	consanguinity
19	F	38	S	non-recordable	
20	F	35	AR	subnormal	
21	F	62	S	non-recordable	diabetic
22	F	37	S	non-recordable	
23	F	51	AD	subnormal	
24	F	27	AR carrier	normal	

S - sporadic AD - autosomal dominant AR - autosomal recessive
XL - X-linked.

human antigens and an SI = 3 with bovine antigens as cutoff values, significant differences ($P<0.05$; Chi square test) were found between retinitis pigmentosa patients and controls with bovine rod outer segments and soluble human retinal antigens, but not with insoluble human retinal antigens and bovine uveal pigment granules ($P>0.05$ for both antigen fractions).

Leukocyte migration inhibition- The results of the leukocyte migration inhibition test (migration indices, MI) performed with the leukocytes of retinitis pigmentosa patients and of controls are presented in Fig. 2.

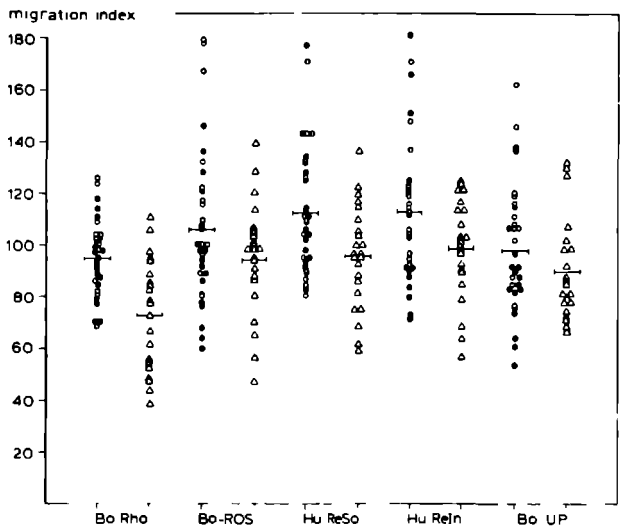

Fig. 2. *Migration of leukocytes of patients suffering from retinitis pigmentosa* (Δ) *and of controls in response to different ocular antigens. The group of the controls consisted of healthy subjects* (o) *and patients suffering from eye diseases other than retinitis pigmentosa* (•). *Horizontal bars indicate the mean migration indices.*

A significant difference (Mann and Whitney U-test) in leukocyte migration was found between retinitis pigmentosa patients and con-

Table II. *Cellular immune response of autosomal dominant (AD) and sporadic (S) retinitis pigmentosa patients.*

Type	Lymphocyte stimulation		Leukocyte migration		
	Bo-ROS	Hu-ReSo	Bo-Rho	Bo-ROS	Hu-ReSo
AD	3.4±2.3(n=10)	2.3±1.1(n=10)	76±26(n=10)	100±12(n=10)	88±16(n=10)
S	3.1±2.7(n=6)	2.0±1.3(n=7)	70±18(n=8)	91±33(n=8)	107±21(n=8)
	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05

Values represent average stimulation indices and migration indices, respectively, with standard errors. The numbers of subjects are given between brackets.

Table III. *Cellular immune response of retinitis pigmentosa patients in relation to ERG response.*

ERG Response	Lymphocyte stimulation		Leukocyte migration		
	Bo-ROS	Hu-ReSo	Bo-Rho	Bo-ROS	Hu-ReSo
Subnormal	3.9±2.6(n=7)	2.2±0.9(n=7)	83±22(n=8)	104±14(n=8)	97±13(n=8)
Non-recordable	3.2±2.3(n=13)	2.0±1.2(n=14)	64±17(n=12)	91±25(n=13)	94±24(n=13)
	P>0.05	P>0.05	P<0.05	P>0.05	P>0.05

Values represent average stimulation indices and migration indices, respectively, with standard errors. The numbers of subjects are given between brackets.

trols after incubation of their leukocytes with bovine rhodopsin ($P = 0.0004$) and soluble human retinal antigens ($P = 0.031$). No significant difference could be observed in migration between the leukocytes of patients and controls incubated with bovine rod outer segments ($P = 0.28$), human insoluble retinal antigens ($P = 0.12$), or bovine uveal pigment granules ($P = 0.14$). Considering an MI = 75% as cutoff value, a significant difference ($P<0.05$; Chi square test) was found between both groups of subjects with bovine rhodopsin and soluble human retinal antigens, but not with the other antigen fractions ($P>0.05$ for these antigen fractions).

The relation between the type of retinitis pigmentosa and the cellular immune response as measured by lymphocyte stimulation and leukocyte migration inhibition is given in Table II. No significant differences (Mann and Whitney U-test) in lymphocyte stimulation or leukocyte migration could be found between autosomal dominant retinitis pigmentosa patients and patients suffering from sporadic forms of retinitis pigmentosa, with either antigen fraction. The numbers of patients with sex-linked retinitis pigmentosa and autosomal recessive retinitis pigmentosa were to small to consider.

Leukocytes of patients with non-recordable ERG responses were significantly more inhibited in their migration by purified rhodopsin than leukocytes of patients with subnormal ERG responses (Table III). No difference in lymphocyte stimulation or leukocyte migration was found between both groups of patients with bovine rod outer segments and soluble human retinal antigens.

Antibody titer- One retinitis pigmentosa patient (8%) and three controls (9%) displayed antibody titers of 40-80 to bovine rhodopsin, rod outer segments, uveal pigment granules and soluble human retinal antigens. An antibody titer in the same range to insoluble human retinal antigens was present in 11 retinitis pigmentosa patients (48%) and 13 controls (37%). These results point to non-specificity of the antibody response or test.

DISCUSSION

The increased concentration of IgM in the serum of some patients suffering from retinitis pigmentosa (21, 25) suggests that (auto)allergic processes are involved in this disease. The observed

immunofluorescence of photoreceptors of rats after covering with sera of retinitis pigmentosa patients (25) supports this. However, the exact nature of the antigen responsible for this reaction could not be established. Immunization of animals by systemic injection of rod outer segments resulted in the destruction of the photoreceptor layer of the retina (27, 10, 29). Moreover, degeneration of the photoreceptors appears to be involved in the pathogenesis of retinitis pigmentosa as well (19). Consequently, it seems reasonable to suggest that, in human beings, an auto-immune response to photoreceptor antigens is manifest during advanced stages of autosomal dominant and sporadic retinitis pigmentosa.

In the present study, delayed-type hypersensitivity in some patients with retinitis pigmentosa could be established by the incorporation of tritiated thymidine into DNA after stimulation of their lymphocytes by soluble human retinal antigens or bovine rod outer segments. The leukocyte migration inhibition test, based on the production of a leukocyte migration inhibition factor by sensitized lymphocytes after contact with the appropriate antigens, partially supported these results. According to Read and Zabriskie (22), the form of the antigen, whether soluble or particulate, is one of the critical factors for accurate functioning of the leukocyte migration inhibition system. This was earlier detected by Zabriskie and Falk (31) using tubercle bacilli as particulate antigen. This phenomenon may explain why bovine rod outer segments are active in the lymphocyte stimulation test and inactive in the leukocyte migration inhibition test. The high stimulatory activity of purified rhodopsin, solubilized by detergent, as measured by the latter technique indicates that this protein may be (one of) the primary antigen(s) responsible for the activation of the cellular immune system of retinitis pigmentosa patients. The high content of rhodopsin in the rod outer segments and the common antigenicity between rhodopsin and soluble retinal antigens (2) may explain the stimulatory activity of these fractions. However, the possibility that the cellular immune response was directed to more than one type of antigen present in these fractions may not be excluded.

The insoluble part of human retinas (devoid of photoreceptor antigens), on the other hand, seems not to be involved in the stimulation of the cellular immune system of these patients.

Previous studies has revealed the antigenicity of bovine uveal pigment granules (3). The failure to detect an immune response to these antigens in patients suffering from retinitis pigmentosa suggests that they are not involved in the pathogenesis of this disease.

The low and probably non-specific antibody titers to most of the tested antigen fractions as found with the complement fixation test suggest that primarily the cellular part of the immune system is involved in retinitis pigmentosa patients. This corroborates the study of Char et al (4) showing an increased lymphocytotoxicity to retinoblastoma cells (probably derived from the photoreceptor layer of the retina) in patients suffering from retinal degenerations, including retinitis pigmentosa, as compared to control persons. Moreover, a strong stimulation especially of the cellular part of the immune system could be demonstrated by injecting purified rhodopsin into rabbits (2). The antigenicity and specificity of rhodopsin has been demonstrated earlier. Homologous rhodopsin, emulsified with an adjuvant, injected into monkeys led to pathological alterations confined especially to the outer segments of the retina (30).

Studies performed on laboratory animals suffering from one type of inherited retinal degeneration revealed an imbalance in renewal and degradation of the rod outer segments resulting in their accumulation in the retina (12, 11). Superficial similarities are present between the animal model and retinal degenerations in human beings (11), although (for instance) the disease in RCS rats differs basically from retinitis pigmentosa in man (16, 23). Accumulation and possible exposure of the strong antigenic rod outer segments to the reticuloendothelial system might evoke an immune response predominantly directed to rhodopsin. The increased exposure of rhodopsin-containing material may break a possible state of immune tolerance. Alternatively, the visual pigment may be a new antigen to the immune system because of its relatively isolated position in the retina. Exposure of the outer segment material to the immune system might be increased by failure of the blood-retinal barrier which is concomitant with the degeneration of rod outer segments in retinitis pigmentosa. An alteration in the primary structure of rhodopsin in patients suffering from retinitis pigmentosa inducing a new antigenic determinant might also be possible. The latter might result in an incapacity of the pigment epithelium cells to phagocytise rod outer segments. In contrast,

animals studies have demonstrated that retinal pigment epithelium cultured from dystrophic rats rarely ingests normal outer segment material (7) possibly by a defect in the pigment epithelium cells. Dowling and Sidman (6) were not able to find any difference in chemical or physical properties between rhodopsin derived from normal and dystrophic rats.

The group of disorders known as retinitis pigmentosa is a classical example of genetic heterogenicity. Retinitis pigmentosa can be transmitted as an autosomal recessive, autosomal dominant or an X-linked recessive trait. The different genetic forms of retinitis pigmentosa have different prognosis for vision (13). It is probable that each genetic form of the disease has a different underlying abnormality (14). We did not obtain indications that the immune response quantitatively or qualitatively varied according to the type of retinitis pigmentosa. It therefore seems likely that the activation of the immune system is a secondary process evoked by rhodopsin containing rod outer segments or other retinal fragments in the affected tissues. The failure to detect a positive immune response in a person known to be carrier of the disease with one of the antigen fractions, points also in this direction. Patients with a non-recordable ERG response reacted significantly more to purified rhodopsin as patients with subnormal ERG response. This suggests that the activation of the immune system depends on the extent of the retinal degeneration. Alternatively, the cellular immune activity found in retinitis pigmentosa patients might account for amplification of retinal degeneration leading to non-recordable ERG responses and accompanying disorders as retinal vascular leakage resulting in oedema.

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CHAPTER 5

COMMON ANTIGENIC DETERMINANTS IN THE SOLUBLE RETINAL FRACTIONS OF MAN, MONKEY, PIG AND CALF

C.J.J. Brinkman, R.M. Broekhuyse and E.F.J. Tolhuizen.

SUMMARY

Common antigenicity between the soluble retinal fractions of man, monkey, pig and calf has been investigated by means of several immunological techniques. Lymphocytes of rabbits sensitised to human retinal antigens are equally stimulated by human, monkey and pig retinal extracts. The results of immunoelectrophoresis point to the presence of immunologically identical antigens in human, monkey and pig retinas. In immunodiffusion, bovine and pig retinal antigens show only a reaction of partial identity with human retinal antigens. In immunoelectrofocusing, no precipitin lines are formed between basic retinal proteins of the tested species and rabbit anti-human retina antiserum. By means of the same technique reactive antigens with an IEP of 4.7 have been demonstrated in the soluble retinal extracts of all investigated species. Both, human and pig retinal extracts contain a reactive antigen with an IEP of 5.3. They equally stimulated the lymphocytes of retinitis pigmentosa patients, which might be attributed to a sensitisation of these patients to this antigen. In human, monkey as well as in bovine retinas an antigenic protein with an IEP of 5.9 was detected.

INTRODUCTION

One of the largest problems in ophthalmic research, is the limited availability of human eye tissues, which makes it necessary to use animal material. However, it is still a matter of uncertainty to what extend animal and human eye tissues are immunologically similar.

Guinea pig macrophages sensitised to bovine retinal antigens

were equally inhibited in their migration by bovine retinal antigens and by retinal extracts from different mammalian species, including man (6). In immunodiffusion experiments, precipitin lines have been obtained with anti-guinea pig retina antiserum or anti-bovine retina antiserum versus the soluble fractions of retinas of rat, bovine, man, pig, guinea pig or sheep (1, 15, 9, 8). These studies suggested immunochemical relationship between retinal proteins of these species.

The present study is an extension of the earlier investigations concerning cross-antigenicity between retinal antigens of different species by using antiserum raised to human retinas. Different immunological techniques have been used to compare the antigenicity of the soluble proteins of human retinas with those of monkey, calf and pig. In this way animal retinal antigens have been detected which may replace human antigens in certain tests.

MATERIAL AND METHODS

Subjects- For lymphocyte stimulation, blood was taken from patients suffering from retinitis pigmentosa, patients with eye diseases not concerning the retina, patients suffering from rheumatoid arthritis and healthy volunteers.

Antigen preparations- Enucleated human eyes were stored in a sterile moist chamber at 4° C. Retinas were dissected from these eyes within 48 hr after enucleation and stored at -70° C. Retinas of monkeys (Macaca fascicularis), pigs and calves were dissected within 8 hr following death and used immediately. The soluble fraction of the retinas was isolated as described previously for human retinas (3). The protein content of the lyophilised soluble extracts of the retinas of man, monkey, pig and calf (defined as Hu-ReSo, Mo-ReSo, Pi-ReSo and Bo-ReSo, respectively) amounted to about 50% (13) using serum albumin as standard.

Immunisation- New Zealand white rabbits of both sexes were used for immunisation. Fourteen adult rabbits were injected with human retinas. Each animal received one retina, suspended in 1 ml phosphate buffered saline (PBS), pH 7.2, emulsified with 1 ml Freund's complete adjuvant. Injections were given subcutaneously at multiple sites on the back and in the groins. For the preparation of antiserum 2 of these rabbits received booster injections, consisting of one human re-

tina suspended in 1 ml PBS without adjuvant, 5 and 7 weeks following the first injection. The antibody titre was monitored by immunodiffusion. Antiserum was isolated one week later.

Lymphocyte stimulation- The isolation of lymphocytes from heparinised human blood has been described (3). Four weeks following the first antigen injection, blood was taken from an ear vein of the rabbits for lymphocyte isolation (5). Human and rabbit lymphocytes were cultured with 100 µg and 250 µg (protein based) of soluble retinal antigens, respectively, from different species. In addition, rabbit lymphocytes were also cultured with 50 µg serum proteins of these species. Lymphocyte activity was measured by the incorporation of ^{3}H-thymidine and was calculated according to the formula for the stimulation index (S.I.). S.I. is the ratio of ^{3}H-thymidine incorporated in cells in the presence of retinal or serum antigens to that incorporated in cells in the absence of retinal or serum antigens.

Immunochemistry- For all immunoprecipitation tests, the retinal fractions were solubilised in PBS to a concentration of 10 mg/ml. The technique of Ouchterlony (14) has been used for immunodiffusion. Preliminary tests were carried out to determine the optimal antigen-antiserum ratio. In order to absorb anti-human serum antibodies, the central well was filled with undiluted human serum which was allowed to diffuse for 1 hr at 37^{o} C into the agarose gel before applying anti-human retina antiserum to the same well. Immunoelectrophoresis was carried out in agarose gels according to the technique described by Scheidegger (16). Anti-human serum antibodies were eliminated as described for immunodiffusion. Rocket immunoelectrophoresis (12) of the soluble retinal fractions of different species has been performed in agarose gel containing anti-human retina antiserum. Immunoelectrofocusing of the retinal fractions of different species has been performed as described for lens proteins (2). For elimination of human serum proteins out of the human retinal fractions and of human serum protein-like determinants out of the animal retinal fractions, anti-human serum antibodies were coupled to CNBr-Sepharose 4B (17). The retinal fractions were purified by affinity chromatography over this adsorbent and the absence of human serum proteins or human serum protein-like determinants was checked by immunodiffusion.

Statistical analysis- The student t-test for paired values was used. Differences between antigen preparations were considered to be

significant at $P < 0.1$.

RESULTS

Lymphocyte stimulation- The average stimulation indices found after incubation of lymphocytes of all human subjects with the soluble retinal fractions of man, monkey, pig or calf (Hu-ReSo, Mo-ReSo, Pi-ReSo and Bo-ReSo, respectively) are presented in Table I.

Table I. *Stimulation of lymphocytes of human subjects with retinal extracts of various species.*

	Antigen fraction			
	Hu-ReSo	Mo-ReSo	Pi-ReSo	Bo-ReSo
All human subjects (36)	1.7 ± 1.4	1.4 ± 0.9	1.5 ± 0.7	2.0 ± 2.0
		P > 0.1	P > 0.1	P > 0.4
Patients reacting positively* (6)	4.1 ± 2.2	1.9 ± 0.9	4.4 ± 4.0	1.7 ± 0.6
		P < 0.02	P > 0.8	P < 0.05

Values represent mean stimulation indices ± S.D. Number of subjects is given between brackets.

* Reacting positively means: S.I. ≥ 2.

Comparing the individual stimulation indices found with Mo-ReSo, Pi-ReSo or Bo-ReSo with the stimulation indices found with Hu-ReSo revealed no differences ($P > 0.1$ for either antigen fraction). Lymphocytes of six patients suffering from retinitis pigmentosa appeared to react positively with Hu-ReSo (i.e. S.I. ≥ 2) and showed an average S.I. of 4.1 with Hu-ReSo and 4.4 with Pi-ReSo (Table I). The individual values between both antigen fractions neither differed significantly ($P > 0.8$). Incubation of these lymphocytes with Mo-ReSo and Bo-ReSo resulted in average stimulation indices of 1.9 and 1.7, respectively. The individual stimulation indices differed significantly from those found with Hu-ReSo ($P < 0.05$ for both antigen fractions). These results suggest a similar stimulative potency of human and pig soluble retinal antigens with respect to human lymphocytes.

The average stimulation indices of lymphocytes of rabbits, immunised with human retinas, after cultivation with soluble retinal fractions of different species are given in Table II.

Table II. *Stimulation of lymphocytes of 14 rabbits, sensitised to human retinal antigens, with retinal extracts and serum proteins of various species.*

	Man	Monkey	Pig	Calf
Retinal extract	8.5 ± 10.2	8.1 ± 9.9 P > 0.7	5.4 ± 6.2 P > 0.1	4.2 ± 3.8 P < 0.1
Serum proteins	1.6 ± 1.4	0.8 ± 0.6	1.5 ± 1.0	1.3 ± 0.8

Values represent mean stimulation indices ± S.D.

Comparing the individual stimulation indices, no statistically significant difference between Hu-ReSo and Mo-ReSo or between Hu-ReSo and Pi-ReSo was observed (P > 0.1 for both antigen preparations). Between Hu-ReSo and Bo-ReSo a difference in stimulatory capacity was present (0.05 < P < 0.1). The rabbit lymphocytes did not react with serum proteins of either species which excludes the influence of these proteins on the observed stimulation by retinal antigens.

Immunochemistry- Immunodiffusion of the soluble retinal fractions of man, monkey, pig and calf versus anti-human retina antiserum revealed two precipitin lines with each antigen fraction (Fig. 1).

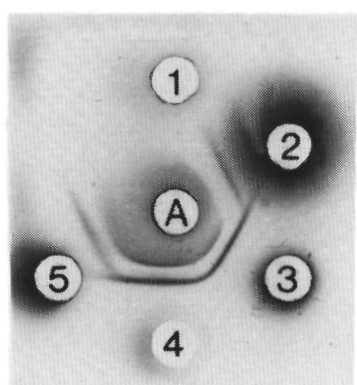

Fig. 1. *Immunodiffusion of rabbit anti-human total retina antiserum (A) versus human serum (1) and versus the soluble retinal fractions of calf (2), man (3), monkey (4) and pig (5). The central well was filled with undiluted human serum for 1 hr at 37° C before applying the antiserum.*

Apart from one common precipitin line closest to the central well, complete fusion was observed between the second precipitin lines of the human and monkey retinal fractions. A reaction of partial identity was found for the second precipitin line of human and bovine retinal antigens, and of monkey and pig retinal antigens. Human serum proteins gave no precipitin lines.

Immunoelectrophoresis confirmed the results of immunodiffusion by showing an almost identical electrophoretic pattern with Hu-ReSo and Mo-ReSo although some difference in mobility was found in the most negatively-charged antigen (Fig. 2(a)).

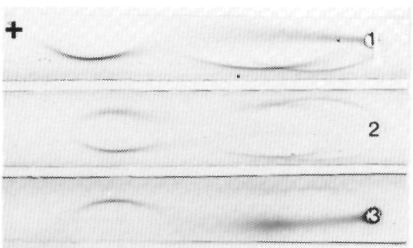

Fig. 2(a).

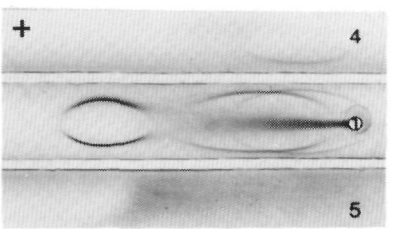

Fig. 2(b).

Immunoelectrophoresis of rabbit anti-human total retina antiserum versus the soluble retinal fractions of man (1), monkey (2), pig (3), calf (4) and versus human serum (5). The trough was filled with undiluted human serum for 1 hr at 37° C before applying the antiserum.

The immunoelectrophoretic pattern of Pi-ReSo was similar to that of Mo-ReSo (Fig. 2(a)). The soluble fraction of bovine retinas formed only one precipitin line (Fig. 2(b)). No precipitin lines were observed with human serum proteins (Fig. 2(b)). Rocket-immunoelectrophoresis of the soluble retinal fractions of the different species demonstrated almost identical precipitin arcs with Hu-ReSo and Mo-ReSo after electrophoresis into the agarose containing anti-human retina antiserum (Fig. 3). A more complex precipitin pattern was observed with Pi-ReSo whereas Bo-ReSo gave only a small precipitin arc. All retinal fractions were used in equal amounts.

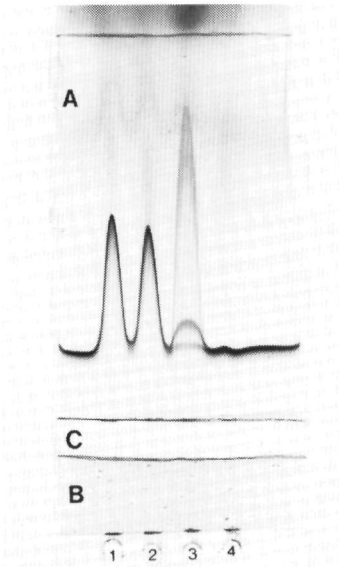

Fig. 3. *Rocket immunoelectrophoresis of the soluble retinal fractions of man (1), monkey (2), pig (3) and calf (4). Part A of the agarose gel contained rabbit anti-human total retina antiserum, part B contained Hu-ReSo and part C consisted of agarose.*

Immunoelectrofocusing of the retinal fractions is demonstrated in Fig. 5. Although the iso-electrofocusing patterns of the various retinal fractions have a somewhat different appearance (Fig. 4), immunodiffusion of these separated proteins versus anti-human retina antiserum revealed the presence of precipitin lines only in the acid pH area (Figs 5 and 6). All retinal fractions contained an antigenic determinant with an isoelectric point (IEP) of 4.7. An antigenic protein with an IEP of 5.3 was present in Hu-ReSo and Pi-ReSo. Hu-ReSo, Mo-ReSo and Bo-ReSo gave immunoprecipitin lines at an IEP of 5.9. The retinal fractions of the various species used in this test contained no serum proteins after affinity chromatography, as checked by immunodiffusion. Therefore, no serum protein bands or precipitin lines are present in the (immuno) electrofocusing patterns.

Fig. 4. ***Iso-electrofocusing*** *of the soluble retinal fractions of man (1), monkey (2), pig (3) and calf (4).*

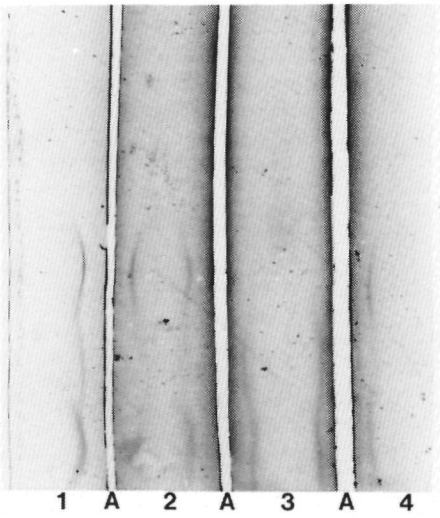

Fig. 5. *Immuno-electrofocusing of the soluble retinal fractions of man (1), monkey (2), pig (3) and calf (4) versus rabbit anti-human total retina antiserum (A).*

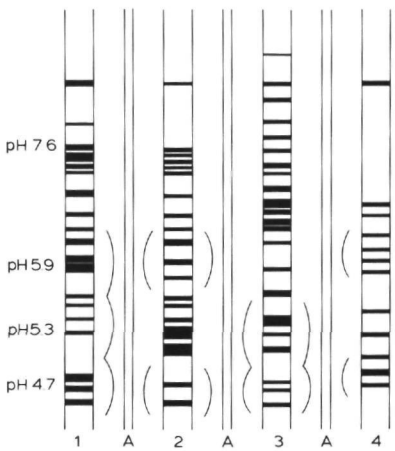

Fig. 6. *Drawing of the pattern of Fig. 4 and Fig. 5. Widths of the bands are not representative for the real amounts of protein. It was intended to show only the position of the bands and the precipitin lines. Reactions of (partial) identity have not been indicated.*

DISCUSSION

There exists a common antigenicity between retinal extracts of different mammalian species (1, 15, 6, 9, 8). Experimental uveitis in rabbits and guinea pigs and in monkeys, however, could only be induced following immunisation with homologous retinovitreous (1) or retinal

extracts (18) indicating the presence of a species specific retinal antigen. The discriminative potentiality of the immunodiffusion technique which was used in most of the in-vitro studies concerning cross-antigenicity between the retinal extracts of different species, is rather low. Species specific reactions might be overlooked due to overwhelming immune responses of non-species specific retinal antigens. In order to obtain more specific information concerning the common antigenicity between retinal proteins of man and various animal species we have tested anti-human retina antiserum with different immunological techniques.

Similar results were obtained concerning the antigenic relationships between the soluble retinal fractions of man and monkey by four different immunochemical techniques. Immunoelectrofocusing revealed that an antigenic protein with an IEP of 5.3 which was present in the human soluble retinal fractions was lacking in the retinal fraction of Macaca fascicularis. The soluble fraction of pig retinas resembled, from the immunological point of view, more to human retinal antigens as compared to soluble bovine retinal antigens. Stimulation of rabbit lymphocytes, sensitised to human retinal antigens, was in accordance with the results of the immunoprecipitation techniques. Similar activation of these rabbit lymphocytes was registered after incubation with human, monkey or pig retinal antigens, whereas bovine retinal fractions gave significantly different results. In addition, the results showed the sensitivity of some patients suffering from retinitis pigmentosa to human soluble retinal antigens. These (unexpected) findings have further been described elsewhere (4). Lymphocytes of these patients reacted to the same extend with human and pig retinal antigens, whereas monkey and bovine antigens were less active. This suggests that patients suffering from retinitis pigmentosa may become selectively sensitised to certain retinal antigens which are also present in the soluble retinal fraction of pigs. These retinal antigens may be represented by precipitin lines at IEP 5.3 found in immunoelectrofocusing of human and pig retinal antigens versus anti-human retina antiserum (Figs. 5 and 6). This may explain why lymphocytes of patients with retinitis pigmentosa reacted less to bovine retinal antigens as did lymphocytes of some patients suffering from retinal detachment. The latter could be stimulated to similar or higher degree with bovine retinal antigens as compared to human retinal antigens

(3). Attempts have been undertaken to determine the nature of different retinal fractions of the same species more precisely. A pathogenic retinal antigen (S-antigen) with a molecular weight of about 55 000 was isolated from retinas of guinea pigs (20) and calfs (21). This antigen seems also to be present in the retina of other species including man (8) with slight variation in the isoelectric point, which range from 5.0 to about 6.1. It is conceivable that the observed common precipitin line found with immunoelectrofocusing of human and pig retinal antigens is the same pathogenic S-antigen.

Our results of immunoelectrofocusing demonstrated the presence of another two antigenic components, having an IEP of 4.7 and 5.9, respectively, in the soluble fraction of human retinas. None of the soluble retinal fractions of these species formed a precipitin line with the anti-human retina antiserum in the region of IEP 6.5 to 9.0, despite the fact that several protein bands were present in this region. Possibly, this is the result of weak immunogenicity of predominantly basic human proteins in the rabbit. Structural resemblance between the concerning human and rabbit proteins and/or antigenic competition could be the causes.

The comparative study of ocular antigens of different animal species and man may be important in view of the therapy of certain eye diseases. The immunopathogenicity of retinal antigens has been described in guinea pigs (22, 1), rabbits (1) and monkeys (18). These findings have been confirmed and extended in several laboratories. Specific retinal antigens probably play a dominant role in the pathogenesis of some forms of chorioretinitis (19). Cell-mediated immune activity could be detected with different retinal preparations in some patients suffering from chorioretinitis (10, 11). The study of de Kozak et al. (7) demonstrated the inhibition of uveo-retinitis in guinea pigs after repeated injections with homologous retinal antigens. Active immunisation of patients suffering from chorioretinitis with appropriate retinal antigens might similarly result in improvement. Possibly, these retinal antigens can be isolated in sufficient amounts from animal retinas containing reactive antigens identical to those of human retinas.

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CHAPTER 6

OCULAR ANTIGENS X. BOVINE RHODOPSIN AND ALPHA CRYSTALLIN: A STUDY OF THEIR IMMUNOGENICITY IN THE RABBIT AND ANTIGENIC RELATIONSHIPS WITH VARIOUS OCULAR TISSUES.

C.J.J. Brinkman, M.P.J. Oerlemans-van Zutphen and R.M. Broekhuyse.

SUMMARY

Rabbits were sensitised with highly purified bovine alpha crystallin and rhodopsin. Without boosting, alpha crystallin predominantly stimulated the humoral immune system, and rhodopsin predominantly the cellular immune system.

The results obtained by lymphocyte stimulation tests, skin tests and immunodiffusion point to the presence of alpha crystallin determinants in bovine iris and probably in choroid, but not in cornea and retina. Rhodopsin determinants have been detected in cornea and sclera, while they seem to lack in lens and iris.

INTRODUCTION

The knowledge of cross-reactivity between intra-ocular tissues may be helpful in the explanation of the complex immunopathological phenomena sometimes encountered in ophthalmology.

For the investigation of common antigenicity between ocular tissues we have previously used tissue antigens, free of serum proteins (4). This eliminated most of the problems concerning a-specific reactions.

For the present study we have chosen rhodopsin and alpha crystallin, which are both also free of serum proteins. The strong antigenicity of heterologous alpha crystallin in rats and rabbits has been demonstrated (1, 21). Monkeys injected with purified homologous rhodopsin developed a strong immune response leading to irreversible

blindness (27) indicating the strong antigenicity of this lipoglycoprotein.

This report describes the immune response following injection of bovine rhodopsin and alpha crystallin into rabbits. The evoked immune activity has been used to estimate a possible common antigenicity between these antigens and antigens extracted from several eye tissues.

MATERIAL AND METHODS

Antigen isolation- All antigens used in this investigation were isolated from fresh bovine eye tissues (about 2 years age). In order to prevent leaking of lens antigens towards the surrounding ocular tissues, special attention was paid to avoid even the slightest damage to the lens capsule (23). After dissection of cornea and iris, the lens was isolated by rupturing the zonule fibers with scissors. Sclera, choroid and retina were isolated from eyes from which the anterior segment (including the lens) had been removed. Rhodopsin (a gift of Dr. W.J. De Grip, Nymegen) was isolated and purified as described by De Grip, Daemen and Bonting (8). The A_{278}/A_{500} ratio of the rhodopsin preparation amounted to 1.6. Phospholipids and proteins other than rhodopsin were not detectable (24). Alpha crystallin was isolated by applying 80 mg bovine lens crystallins to a Sepharose 6B column (2). The column was eluted with 0.1 M-ammonium acetate buffer, pH 8.0, supplemented with 0.5% butanol. The alpha crystallin fraction was lyophilised and the product was checked for purity by isoelectric focusing (2) and immunodiffusion (Fig. 2). The soluble fractions of sclera, iris and retina were isolated as previously described (4). The soluble corneal and choroidal antigens were prepared in the same way as scleral and retinal antigens, respectively. The soluble lens fraction was prepared by homogenisation of decapsulated bovine lenses in excess of 0.01 M-ammonium acetate buffer, pH 7.3, at 4° C, followed by centrifugation at 15 000 x g for 30 min. at 4° C. The supernatant was lyophilised. All antigen preparations were stored at -20° C until used. The soluble antigen fractions of bovine heart, lung, liver and kidney tissues were isolated as retinal antigens.

Immunisation- Five New Zealand rabbits received 2 mg bovine rhodopsin in 1 ml phosphate buffered saline (PBS), pH 7.2, emulsified with 1 ml Freund's complete adjuvant (FCA) subcutaneously at various sites on the back and in the groins. Boosters were given of 2 mg rhodopsin mixed with 0.5% Tween 80 in 1 ml PBS (without adjuvant) 6, 10 and 13 weeks following the first injection. The same immunisation scheme was performed in 5 rabbits with bovine alpha crystallin using amounts of 5 mg. Antiserum was obtained 7 days after the last injection. Eight rabbits, injected once with FCA, served as controls.

Lymphocyte stimulation- At different times, 15 ml blood was withdrawn from the marginal ear vein and anti-coagulated with heparin. Some blood was used for plasma preparation and the remainder diluted with an equal volume of Eagle's Minimum Essential Medium (MEM). Isolation, culturing, labeling and harvesting of the lymphocytes was carried out as described previously (5). Stimulation of the lymphocytes was done with 500 µg (dry material) tissue antigen, solubilised in MEM at a concentration of 10 mg/ml and sterilised by sonication. Fifty µg of bovine serum protein and 1 mg (dry material) of scleral antigen were used. The degree of stimulation is reported as the stimulation index (S.I.) which is the ratio of the ^{3}H-thymidine incorporation into the lymphocytes in the presence of antigen to that in the absence of antigen.

Skin test- Skin tests were done by injecting 50 µl antigen solution (2 mg dry material per ml PBS) intradermally into the back of the immunised rabbits. The reactions were recorded 4, 24, 48 and 72 hours later and graded according to the following criteria: 0, erythema, induration < 5 mm diameter; +, erythema, induration 5-10 mm diameter; ++, erythema, induration 10-15 mm diameter; +++, erythema, induration > 15 mm diameter.

Immunodiffusion- Immunodiffusion was performed using 1% agarose in 0.1 M-Tris-Veronal buffer at pH 8.8. The antigens were solubilised in PBS at a concentration of 10 mg dry weight per ml. Preliminary tests were done in order to determine optimal antigen-antiserum ratio.

Antibody titre- The anti-alpha crystallin antibody titre was determined by the hemagglutination test as described by Herbert (13)

using 1 ml 2% (^{v}/v) tanned sheep erythrocytes incubated with 1 mg alpha crystallin. The anti-rhodopsin antibody titre was determined by the complement fixation test (22) using veronal buffered saline (0.005 M, pH 7.3) in stead of triethanolamine buffered saline.

RESULTS

Immune response.

Four weeks following the first injection and 2 weeks after the last injection the alpha crystallin-and rhodopsin-sensitised rabbits received an intradermal injection with alpha crystallin and rhodopsin, respectively. The skin reactions were observed 4, 24, 48 and 72 hours later. The results are demonstrated in Table I.

Table I. *Skin reactions of alpha crystallin- and rhodopsin-sensitised rabbits.*

Injected antigen		Intensity of reaction after various periods (hrs).			
		4	24	48	72
Alpha crystallin	before boosting	++	++	+	+
	after boosting	++	+	+	+
Rhodopsin	before boosting	0	+	+	0
	after boosting	++	+++	++	+

Average reactions are represented, graded according to the criteria as described in Materials and methods.

Alpha crystallin-sensitised rabbits showed, before boosting, an immediate-type reaction indicating the presence of anti-alpha crystallin antibodies. After boosting a less severe reaction was observed at 24 hours. The rabbits sensitised to rhodopsin showed a most intense reaction between 24 and 48 hours, suggesting the presence of a delayed-type hypersensitivity state. Before boosting no reaction could be observed after 4 hours indicating a very low or zero anti-rhodopsin antibody titre.

Common antigenicity.

1. Lymphocyte stimulation- In preliminary studies (5) it was shown that boosting of rabbits decreased the cellular immune response to total corneal epithelium antigens as determined by the lymphocyte stimulation test. In order to determine common antigenicity between alpha crystallin or rhodopsin and the soluble antigens extracted from various eye tissues, the lymphocyte stimulation test was performed 4-5 weeks after the first antigen injection.

Alpha crystallin- Rabbit lymphocytes sensitised to alpha crystallin were cultured with the soluble antigens from isolated eye tissues and with bovine serum. As demonstrated in Table II, with lens and scleral antigens, a significant difference in lymphocyte activity between alpha crystallin-sensitised rabbits and rabbits injected with FCA ($P < 0.05$; Wilcoxons' two sample test) could be demonstrated.

Rhodopsin- The data presented in Table III show that rabbit lymphocytes sensitised to rhodopsin could be stimulated with the soluble antigens extracted from cornea, sclera and retina. The differences in S.I. between rhodopsin-sensitised rabbits and FCA-sensitised rabbits were significant for these antigens ($P < 0.05$).

Lymphocytes of rabbits sensitised to rhodopsin or alpha crystallin could not be stimulated with the soluble fractions of bovine heart, lung, liver or kidney tissues.

2. Skin test- Ten days after the last booster injection all rabbits received an intradermal injection with the soluble fractions of eye tissues and with bovine serum. The skin reactions of the rabbits sensitised with alpha crystallin and rhodopsin were maximally 4 and 24 hours, respectively, after injecting the antigens. The alpha crystallin-sensitised rabbits developed a positive skin reaction not only with alpha crystallin but also with the soluble antigen fractions of lens, iris and sclera. Rabbits sensitised with rhodopsin developed a positive skin reaction with all antigen fractions with the exception of lens antigens, iris antigens and bovine serum. The intensities of the skin reactions are demonstrated in Table IV.

3. Immunodiffusion- The anti-alpha crystallin antiserum and the anti-rhodopsin antiserum with the highest antibody titre (25000 and 1200, respectively, as determined by the hemagglutination test

Table II. *Stimulation of lymphocytes of rabbits sensitised with alpha crystallin and of FCA - sensitised rabbits with soluble antigens extracted from eye tissues and with bovine serum.*

Injected antigen	Cornea	Lens	Iris	Retina	Choroid	Sclera	Bovine serum
Alpha crystallin	2.1±0.8	6.1±6.8*	2.4±3.5	1.5±0.5	1.2±0.5	2.7±2.5*	1.1±0.5
FCA	1.2±0.8	1.3±0.6	0.6±0.2	1.1±0.3	0.8±0.3	0.6±0.4	1.4±0.7

Values represent average stimulation indices ± S.D.

* Differences between alpha crystallin-sensitised rabbits and FCA-sensitised rabbits statistically significant ($P < 0.05$).

Table III. *Stimulation of lymphocytes of rabbits sensitised with rhodopsin and of FCA - sensitised rabbits with soluble antigens extracted from eye tissues and with bovine serum.*

Injected antigen	Cornea	Lens	Iris	Retina	Choroid	Sclera	Bovine serum
Rhodopsin	5.1±2.8*	1.8±0.9	1.4±1.1	5.1±2.5*	2.2±1.6	1.5±0.9*	1.5±0.6
FCA	1.2±0.8	1.3±0.6	0.6±0.2	1.1±0.3	0.8±0.3	0.6±0.4	1.4±0.7

Values represent average stimulation indices ± S.D.

* Differences between rhodopsin-sensitised rabbits and FCA-sensitised rabbits statistically significant ($P < 0.05$).

Table IV. *Skin reaction of rabbits sensitised with alpha crystallin and of rabbits sensitised with rhodopsin after intradermal injection of soluble antigens of eye tissues and bovine serum.*

Injected antigen	Alpha crystallin	Rhodopsin	Cornea	Lens	Iris	Retina	Choroid	Sclera	Bovine serum
Alpha crystallin[a]	++	n.r.	0	+++	+	0	0	+	0
Rhodopsin[b]	n.r.	+++	+	0	0	+	+	+	0

[a]Maximal skin reaction as recorded at 4 hours; [b]Maximal skin reaction as recorded at 24 hours; n.r. = not recorded.

and the complement fixation test) were used for a closer investigation into the common antigenicity between these proteins and antigens extracted from other tissues of the eye and bovine serum. Optimal antiserum-antigen ratios were determined in preliminary experiments. From these experiments it appeared that no precipitin line developed between anti-rhodopsin antiserum and the ocular antigens or bovine serum. Anti-rhodopsin antiserum versus rhodopsin (2 mg/ml in 2% digitonin) did form precipitin lines. Anti-alpha crystallin antiserum formed clear precipitin lines with the antigens extracted from iris, lens and choroid (Fig. 1). The fusion of these precipitin lines suggests the presence of at least one common antigen.

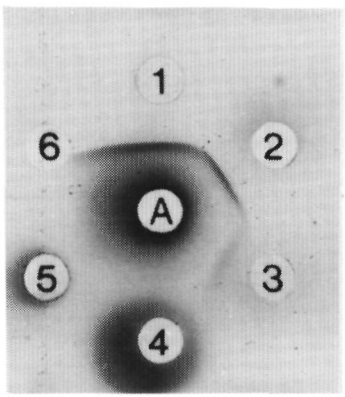

Fig. 1. *Immunodiffusion of rabbit anti-bovine alpha crystallin antiserum (A), versus the soluble antigen fractions of lens (1), iris (2), choroid (3), retina (4), sclera (5) and cornea (6).*

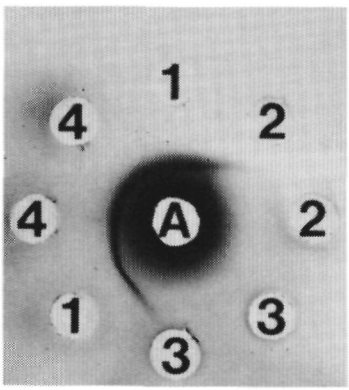

Fig. 2. *Immunodiffusion of rabbit anti-bovine alpha crystallin antiserum (A), versus alpha crystallin (1), beta crystallin (2), gamma crystallin (3) and total lens crystallins (4). The antigens have been applied in two different concentrations.*

Fig. 2 shows the results of immunodiffusion of anti-alpha crystallin antiserum versus alpha-, beta- and gamma crystallins and total lens crystallins. Proteins other than alpha crystallin were beyond the limit of detection.

The results of lymphocyte stimulation, skin test and immunodiffusion are summarised in Table V.

Table V. *Summary of results of lymphocyte stimulation (LST), skin test (ST) and immunodiffusion (ID) performed on alpha crystallin- and rhodopsin-sensitised rabbits with soluble antigens of eye tissues.*

		Cornea	Lens	Iris	Retina	Choroid	Sclera
	LST	-	+	-	-	-	+
Alpha crystallin	ST	-	+	+	-	-	+
	ID	-	+	+	-	+	-
	LST	+	-	-	+	-	+
Rhodopsin	ST	+	-	-	+	+	+
	ID	-	-	-	-	-	-

+ positive response; - negative response

DISCUSSION

Apart from the route of immunisation, the presence of an adjuvant and the dose of material, the response of the immune system depends on the physical state of the antigens. It is believed that insoluble antigens, in isolated form or as transplants (membrane antigens), predominantly stimulate the cellular immune system by activation of T-lymphocytes. Soluble antigens, on the other hand, lead to a more clear response of the humoral immune system by activation of B-lymphocytes (20). The results of the present communication are in accordance with this assumption. Rhodopsin predominantly stimulated the T-lymphocyte system after being injected into rabbits. This was demonstrated by the skin reaction which showed a maximum intensity 24-48 hours after intradermal rhodopsin injection. Immunisation of rabbits with the soluble antigen alpha crystallin led to a different immune response. Before boosting a strong skin reaction was observed 4 hours following intradermal alpha crystallin injection indicating the presence of anti-alpha crystallin antibodies. Boosting these rabbits with alpha crystallin resulted in an increase of the antibody titre but not of the cellular immune response. Other investigators have found similar results. Immunising rats with soluble bovine albumin resulted in good antibody formation while polymerised (insoluble) bovine serum albumin predominantly activated the cellular

immune system (15). The overwhelming response of the humoral immune system after injection of alpha crystallin may partially be caused by the chemical structure of this protein. Bovine alpha crystallin predominantly consists of 4 major, partially identical, subunits (11), which might render it thymus-independent like polymerised flagellin (7).

The difference in immune response following injection with alpha crystallin and rhodopsin has forced us to use different immunological techniques for the investigation of a possible common antigenicity between these proteins and antigens extracted from other bovine ocular tissues.

Alpha crystallin-sensitised rabbit lymphocytes were stimulated after incubation with lens and scleral antigens. Skin testing (which can be a more sensitive technique than lymphocyte stimulation (6)) of the alpha crystallin-sensitised rabbits revealed a positive reaction with sclera, iris and lens antigens. These results were partially supported by immunodiffusion which demonstrated cross-reactivity between alpha crystallin and antigens from iris, choroid and lens. Because alpha crystallin evoked a predominantly humoral immune response in our experiments, we conclude that (outside the lens) determinants of this protein are present in the iris. Probably they are also present in the choroid, although a confirmative skin test was lacking. The negative results of the lymphocyte stimulation test can be explained by the very low or absent cellular immune response. The purity of the alpha crystallin preparation (Fig. 2) eliminates the possibility that the observed common antigenicity was caused by proteins other than alpha crystallin. No precipitin line was found between scleral antigens and anti-alpha crystallin antiserum. Therefore a further study concerning these antigens is needed.

Our results partially confirm the studies of Maisel and Harmison (18) who isolated an antigen from the chick iris which was immunologically and electrophoretically identical to alpha crystallin, and of Bours and van Doorenmaalen (3), who demonstrated common antigenicity between alpha crystallin and soluble extracts of the cornea, vitreous body, aqueous humor, iris and retina of chick eyes. With bovine tissues, we were not able to demonstrate common antigenicity between alpha crystallin and soluble antigens from cornea and retina. Immunofluorescence studies of one-day old rat eyes (19) and radio-

immunoassays of sheep eye extracts with anti-alpha crystallin antiserum (23) did not reveal alpha crystallin determinants outside the lens. The discrepancy with respect to our results may originate from technical as well as from species differences, but are as yet difficult to explain.

Phacoanaphylactic endophthalmitis is, by some authors, considered to be the result of an autoimmune response to alpha crystallin (17, 10). The presence of alpha crystallin determinants in the iris and probably in the choroid as demonstrated in this study is in agreement with this hypothesis, although we have to keep in mind that non-alpha crystallin lens determinants have also been found to be common to various ocular tissues, including the iris (16).

By use of lymphocyte stimulation and skin test it was demonstrated that the soluble antigens extracted from cornea, retina and sclera stimulated the rhodopsin-sensitised lymphocytes suggesting cross-antigenicity between these tissues and rhodopsin. The sera of the tested rhodopsin-immunised rabbits displayed a to low humoral antibody activity to yield precipitin lines with the various eye tissue extracts. This was due to the ability of rhodopsin to evoke a cellular immune response as has been discussed above. The observed skin reaction to choroidal antigens has to be considered with some caution because it is known that uveal tissues are difficult to dissect without contamination with retinal fragments (25). For this reason we cannot draw any conclusion from this result. The absence of stimulation of rhodopsin-sensitised lymphocytes by bovine serum or antigens extracted from extra-ocular tissues eliminated the influence of serum proteins or histocompatibility antigens on these results.

Besides the previously detected strong immune response to purified homologous rhodopsin after injection into monkeys (27), partially purified rhodopsin and rod outer segments induced chorioretinal damage in guinea pigs after injecting into footpads (9). Systemic immunisation of monkeys (26) and rabbits (12) with rod outer segments resulted in ocular inflammation. Patients suffering from chorioretinitis seem to develop an autoimmune response to rod outer segments in some cases (14). In view of the presence of considerable amounts of rhodopsin in the rod outer segments, this autoimmune response may be directed to rhodopsin. Therefore, the common antigeni-

city between rhodopsin and other intra-ocular tissues as determined in the present study could explain the complex reaction sometimes encountered in chorioretinitis.

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The anatomical partition of the cornea in epithelium, stroma and endothelium seems partially to be reflected in a difference in antigenic composition and immunogenicity of these components. In addition to shared antigens (31), each corneal layer includes one or more layer-specific antigens (3, 31). Suggestions have been made that the typical patterns of rejection of the peculiar layers of the cornea, sometimes observed after penetrating corneal transplantation (11), may be based on these layer specific antigens. Alternatively or in addition, differences in rejection patterns may be based on the typic anatomical architecture of the corneal layers. The endothelium borders the aqueous humor and is separated from the stroma by a basement membrane (Descemet's membrane). The stroma is confluent with the sclera from where serum proteins have access to this layer. The epithelium is separated from the stroma by a basement membrane-like layer and has a high regenerative capacity. Antibodies to corneal epithelium (5, 23) and to conjunctival epithelium (5) as the manifestation of an immune response could be demonstrated in patients suffering from Mooren's ulcer, a disease affecting the peripheral part of the cornea. Mondina et al. (17) reported on circulating antibodies to corneal epithelium in a patient with corneal erosions and sterile stromal ulcerations. These findings may partially be explained with the complex protein content of the epithelial part of the cornea (2).

Keratoplasty - An increase in serum immunoglobulin-M level in 13 patients with clinically visible immune reactions to their corneal transplant (33), suggests the involvement of the humoral immune system in keratoplasty. Anti-cornea antibodies were found after keratoplasty in patients suffering from keratoconus who were negative before operation (1). No relation, however, was found between the presence of antibodies and the incipience of graft opacification. Grunnet et al. (7) demonstrated the presence of circulating anti-

cornea antibodies in only 1 out of 25 recipients of a penetrating corneal graft while cytotoxic lymphocytes were present in 8 patients. Six of these 8 patients presented clinical signs of graft rejection suggesting the participation of cellular immunity in some cases after corneal transplantation.

Numerous investigators assume that cellular immunity is involved in the pathogenesis of corneal graft rejection. However, the number of publications dealing with this topic is rather scarce. Intracorneal injection of bovine gamma globulin in rabbits resulted in the production of sensitised T-lymphocytes in the draining preauricular and cervical lymph nodes (26). Delayed-type sensitivity-like reactions could be evoked in the corneas of guinea pigs by intracorneal injection of lymphokines (6). Corneal grafting or intradermal injection of corneal homogenates, in guinea pigs resulted in delayed-type hypersensitivity responses as judged from skin tests, lymphocyte stimulation and lymphokine production (29).

Scanning electron microscopic and histopathologic studies revealed the participation of lymphocytes in the process of corneal graft rejection (18, 19). However, the presence of plasma cells and granulocytes in the stroma and epithelium of a transplanted human cornea in a rabbit detected by light microscopy (32) and electron microscopy (21) indicates that a humoral immune response is committed in the immunological rejection mechanism.

Passive transfer of sensitised lymphocytes has been performed upon rabbits after allografting or xenografting with different results. Whereas Khodadoust and Silverstein (12) described a rejection of penetrating corneal grafts after transfer of specifically sensitised lymph node cells, in the experiments of Meyer and Dietrich (15) and Meyer and Salehi (16), the transfer of lymphocytes did not result in rejection of corneal allografts in rabbits.

The presence of a significant level of macrophage migration inhibition factor in the aqueous humor in the course of xenograft and allograft rejection (24) seems to support the hypothesis of the commitment of cellular immunity in keratoplasty. The possibility that macrophage migration inhibition was mediated by antigen-antibody complexes, however, may not be ruled out in this case according to the authors. Szabo et al., (27) examined in 26 cases of keratoplasty the role of sensitised lymphocytes by lymphocyte stimulation and leuko-

cyte migration inhibition. No lymphocyte activity could be detected upon incubation with soluble corneal antigens while positive results were found with the leukocyte migration inhibition test. These findings were partially in agreement with the results of lymphocyte stimulation tests performed on a group of patients tested both before and after corneal transplantation in our laboratorium. No increase in the number of patients sensitive to soluble human corneal antigens could be detected after operation (28).

Herpes simplex virus keratitis - There is experimental and clinical evidence to suggest that herpes simplex virus keratitis is the result of a delayed-type hypersensitivity reaction in the cornea. Subcutaneous injections of live herpes simplex virus in rabbits resulted in a significant increase in the incidence of disciform keratitis as compared to animals that had not been injected (30). The successful transfer of a state of delayed-type hypersensitivity to herpes simplex virus in guinea pigs by means of leukocytes but not by serum agrees with earlier observations (13). Polack et al., (20) demonstrated clearly that in rabbits sensitisation to herpes simplex virus plays an important role in graft opacification. Grafting of locally herpes simplex virus-sensitised corneal tissues in systemically virus-sensitised recipients resulted in 100% of all cases in corneal opacification within 2 weeks. A pronounced lymphocytic and plasma cell infiltration at the limbus region was present. Transplants from systemically virus-sensitised donor rabbits in locally virus-sensitised hosts led in 50% of all cases to opacification. This last observation was an important finding because it suggests that viral antigens had reached the cornea of the donor animal after systemic sensitisation. Histological and electron microscopic examination of rabbit corneas from experimental animals with disciform keratitis and oedema, following viral inoculation, revealed the presence of numerous lymphocytes and other immunologically active cells (14). The lymphocytes were frequently found in close contact with keratocytes. Immunofluorescent labeling of sections of rabbit corneas obtained from animals with disciform oedema showed viral antigens in keratocytes of the stroma but not in the epithelium. Modification of the cell membrane of rabbit corneal cells (type of corneal cells not mentioned) by incorporation of viral antigens has been de-

monstrated earlier (10). Viral antigens-sensitised lymphocytes, which have been demonstrated in viral-injected human beings by lymphocyte stimulation as well as by leukocyte migration inhibition (22), may contact the viral antigens incorporated in the membranes of the corneal cells. This contact may result in corneal cell destruction followed by liberation of corneal specific antigens. The latter can activate the immune system leading to the presence of circulating corneal specific antigens-sensitised lymphocytes, which could be detected by leukocyte migration inhibition (8, 9, 25) or by lymphocyte stimulation (4).

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CHAPTER 7

Ocular antigens VIII: assay of delayed hypersensitivity to corneal epithelium in the rabbit

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SUMMARY Rabbits were sensitised with complete bovine corneal epithelium The lymphocyte stimulation test was performed with the lymphocytes of these rabbits using the soluble and sonicated insoluble fraction of the corneal epithelium as the antigens A striking difference existed in the optimal test conditions for these antigen fractions

By comparing the results of the lymphocyte stimulation test with other immunological parameters, namely, skin test reaction, antibody titre and phytohaemagglutinin stimulation of the lymphocytes, we concluded that both antigen fractions stimulate predominantly the T-lymphocyte system, although boosting augmented the humoral immune response Stimulation of the cultured lymphocytes by both the separate and mixed antigen fractions is evidence for the existence of cross-reacting antigens between the soluble and insoluble epithelial fractions

It is generally accepted that T-lymphocyte cytotoxicity is the principal effector of corneal graft rejection By means of the leucocyte migration inhibition test as a detector of delayed hypersensitivity cellular immunity to corneal antigens has been established in several disorders of the cornea and after keratoplasty (Henley *et al*, 1971, Shore *et al*, 1972, Henley and Okas, 1974, Szabo *et al* 1975) Moreover, it has been shown that antigens extracted from the whole cornea can elicit cellular immunity in guinea pigs (Ugrinski and Kirkpatrick, 1974)

Most of these studies were performed with soluble antigens extracted from the whole cornea In addition to the intrinsic antigens of the cornea there are appreciable amounts of serum proteins especially localised in the stroma (Broekhuyse, 1972) These serum proteins, with the exception of an albumin-like protein, are absent in the epithelium of the cornea (Berger, 1969), which makes the epithelium suitable for a study of the immunological properties of corneal tissues The possible role of the insoluble fraction of the corneal tissues, including cell membranes and connective tissue, needs further investigation This fraction contains various antigens including the membrane-associated histocompatibility antigens and structural glycoproteins The latter appeared to display transplantation antigen activity (Treffers and Broekhuyse, 1977) Whether sensitisation with xenogenic mixtures supplies adequate information about allogenic transplantation is doubtful, because this transplantation largely depends on histocompatibility antigens. We have, however, carried out such sensitisation in the present study as a preparatory investigation into the nature of the antigenicity of soluble and insoluble (membranous) epithelial constituents of the cornea

After determining the optimal conditions for the lymphocyte stimulation test we compared the antigenicity of both the soluble and the particulate antigens of the corneal epithelium and correlated these results with other immunological parameters, namely, the antibody titre, the skin test, and phytohaemagglutinin stimulation

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Material and methods

ISOLATION OF THE EPITHELIAL ANTIGENS

Fresh calf eyes were washed with saline, dipped in 0 25% chloramphenicol, and transported on ice to the laboratory All further procedures were carried out at 0 to 4 C unless indicated otherwise After rinsing the cornea with saline only the central part of the epithelium was scraped off to avoid contamination with other tissue Homogenisation was carried out in a Potter-Elvehjem homogeniser in 0 01 M ammonium acetate buffer (pH 7 2), the

mixture was sonicated until a homogeneous suspension was obtained For separating the soluble and insoluble fraction the homogenate was centrifuged at 15 000 *g* for 30 min The supernatant was lyophilised and the soluble epithelial fraction (EpSo) was stored at -20 C until used The sediment was washed once with 0 01 M ammonium acetate buffer and several times with water The sediment was then lyophilised and the product the insoluble epithelial fraction (EpIn), was stored at 20 C until used

SENSITISATION

Seventeen adult New Zealand rabbits of both sexes were injected subcutaneously with sonicated complete epithelium (100 mg wet weight dispersed in 1 ml phosphate buffered saline (PBS) and emulsified with 1 ml complete Freund s adjuvant (FCA)) After 1 week 8 rabbits received another identically prepared injection subcutaneously in order to stimulate further antibody response Three rabbits were injected subcutaneously with 15 mg EpSo which had been solubilised in 1 ml PBS and emulsified with 1 ml FCA Skin tests were carried out by injecting 0 05 ml EpSo solution in PBS (5 mg dry weight per ml) The reaction was recorded at 24 hours Unsensitised rabbits were used as controls

LYMPHOCYTE CULTURE

Blood was withdrawn from an ear vein or from the heart and defibrinated with glass beads Lymphocytes were isolated from the diluted blood as described by Du Bois *et al* (1973) 10^6 lymphocytes were suspended in 1 ml Eagle s minimal essential medium (MEM) buffered with 0 025 M Tris (pH 7 4) this solution was supplemented with 100 U ml penicillin 100 ug ml streptomycin, and 15 % homologous inactivated rabbit serum The lymphocytes were stimulated with 50 ug PHA-P (Difco), and with varying amounts of EpSo and EpIn The EpSo and EpIn solutions were sterilised by ultrasonic treatment Sterility was checked by incubating EpSo and EpIn alone in the culture medium

The cells stimulated with PHA-P were harvested after 50 hours and those stimulated with EpSo or EpIn as indicated below under Results Twenty four hours before harvesting 0 5 uCi ^{3}H thymidine (Radiochemical Centre Amersham England, SA 23 3 Ci mmol) was added in 0 02 ml MEM The cells were collected on Millipore glass fibre filters and washed with saline Radioactivity was determined by incubating the filters (after drying by heat) in 6 ml scintillation fluid containing 100 mg dimethyl-POPOP and 5 g PPO in 1 litre toluene Counting was performed in an Isocap 300 scintillation counter (Nuclear Chicago) The results are reported as the stimulation index (SI), which is expressed as SI (cpm of stimulated cells) (cpm of unstimulated cells) A SI 2 is considered to be positive

ANTIBODY TITRE

The antibody titre was determined by the haemagglutination test as described by Herbert (1973) using 1 ml 2 % (w v) tanned erythrocytes incubated with 1 mg antigen (Treffers and Broekhuyse 1977)

Results

CULTURE PERIOD

Table 1 shows the results of ^{3}H-thymidine incorporation into lymphocytes stimulated with 500 ug EpSo and 250 ug EpIn using different culture periods Maximal stimulation indices were achieved between 4 and 6 days for EpSo and between 3 and 4 days for EpIn A period of 5 days for EpSo and a period of 4 days for EpIn were used for further investigations

DOSE RESPONSE

Table 2 shows the results of the ^{3}H-thymidine incorporation into lymphocytes cultured in the presence of various amounts of EpSo and EpIn Mean values of maximal stimulation indices by these fractions occurred at concentrations between 490 ug and 780 ug for EpSo and between 50 ug and 240 ug for EpIn For EpSo a concentration of 500 ug and for EpIn a concentration of 100 ug were considered to be optimal for further investigations

Table 1 *1 10^6 rabbit lymphocytes sensitised with complete corneal epithelium were stimulated with 500 ug EpSo or 250 ug EpIn during varying periods of culture The average period yielding the maximal stimulation index is given*

Average culture period (days) ± SD	
EpSo	*EpIn*
5 0 1 0	3 7 0 75
(n 9)	(n 7)
(range 4 6)	(range 3 5)

Table 2 *1 10^6 rabbit lymphocytes sensitised with complete corneal epithelium were stimulated with various amounts of EpSo and EpIn for 5 days and 4 days respectively Range of dry weight material giving the maximal stimulation index is given*

Range of dry weight material (μg) for maximal SI	
EpSo	*EpIn*
490 780	50 240
(n 9)	(n 6)

Table 3 *Stimulation of corneal-epithelium sensitised lymphocytes with EpSo (5 days cultivation with 500 µg) EpIn (4 days cultivation with 100 µg) EpSo plus EpIn (4 days cultivation with 500 µg EpSo plus 100 µg EpIn) and PHA-P (50 h cultivation with 50 µg) is compared with the antibody titre of EpSo and EpIn and the skin test intensity recorded after 24 h (mm) Stimulation is given as the stimulation index*

Rabbit no	SI					Antibody titre		Skin reaction
	EpSo	EpIn	EpSo	EpIn	PHA-P	EpSo	EpIn	EpSo (ϕ in mm)*
8677	75 3	61 9	73 9		82 9	2 000	4 000	17
8678	19 4	15 0	15 6		30 2	500	100	17
8679	65 7	20 7	22 5		48 6	1 000	2 000	28
8680	81 6	23 9	38 9		152 2	2 000	4 000	26
8681	43 9	24 3	37 5		251 9	4 000	4 000	20
8683	68 0	28 0	18 0		46 2	1 000	500	20
8684	44 0	15 3	36 4		45 1	2 000	4 000	20
8685	69 7	28 5	34 5		52 8	4 000	8 000	26
8686	16 8	16 3	18 1		73 9	2 000	2 000	25
Mean	53 8	26 0	32 8		87 1	2 000	3 000	22

* ϕ represents diameter of erythema

Table 4 *As Table 3 The rabbits received a second dose of antigen emulsified in FC A 1 week after first injection*

Rabbit no	SI				Antibody titre		Skin reaction
	EpSo	EpIn	EpSo	EpIn PHA P	EpSo	EpIn	EpSo (ϕ in mm)*
8	4 5	1 1	2 6	6 7	8 000	16 000	9
9	ND	ND	ND	23 4	2 000	8 000	12
10	10 8	3 8	5 5	50 6	4 000	8 000	10
11	11 1	6 0	7 9	9 3	8 000	8 000	20
13	43 3	10 2	ND	60 4	8 000	4 000	18
14	81 8	9 2	ND	45 1	8 000	16 000	19
15	ND	ND	ND	38 2	4 000	8 000	18
16	17 6	12 2	ND	4 8	4 000	8 000	21
Mean	28 2	7 1		29 8	6 000	9 500	16

* ϕ represents diameter of erythema ND not done

STIMULATION UNDER OPTIMAL CONDITIONS

Table 3 gives the results of experiments in which the lymphocytes from corneal epithelium-sensitised rabbits were tested under optimal conditions A high SI found with EpSo, EpIn, and PHA-P and an intense skin reaction were accompanied by a low antibody production The data in Table 4 are from rabbits which had received a second antigen injection 1 week after the first These rabbits had on the average an antibody titre three times higher than those of Table 3 (P 0 01, Mann-Whitney U test) This high antibody titre was accompanied by a less intense skin reaction (P 0 05) and a much lower SI with EpSo (P 0 09), FpIn (P 0 0005) and PHA-P (P 0 02) than the rabbits shown in Table 3 Statistical analysis of the ratios of SI (EpSo)/antibody titre (EpSo), SI (EpIn) antibody titre (EpIn) and skin reaction (EpSo) antibody titre (EpSo) in Tables 3 and 4 revealed highly significant differences (P 0 0009 P 0 0005, and P 0 0005 respectively)

The lymphocytes of normal rabbits could not be stimulated by FpIn (SI 1 0 0 1, n 4) and sometimes weakly by EpSo (SI 1 5 1 2), n 6) The SI of normal rabbit lymphocytes cultured in the presence of 50 µg PHA-P for 50 hours (optimal circumstances) amounted to 44 0 16 5 (n 10) Normal rabbits showed negative skin reactions to EpSo and negative antibody titres to EpSo and EpIn

Because Tables 3 and 4 show that stimulation of the lymphocytes with EpSo plus EpIn never exceeds the stimulation with EpSo added separately, we investigated the possibility of common antigens in both fractions Table 5 shows that lymphocytes sensitised to EpSo are also stimulated by the EpIn fraction, which means that both fractions contain cross-reacting or possibly identical antigens

Discussion

This investigation shows that it is possible to stimulate corneal-epithelium-sensitised lymphocytes with ultrasonically dispersed cell membranes isolated from the corneal epithelium The particles obtained are vesicles consisting of membrane lipids and proteins The amount of stimulation with the insoluble epithelial fraction was always less than with the soluble epithelial fraction when both were measured under optimal circumstances Two explanations are possible for this phenomenon It is possible that antigens hidden in the vesicle membrane or included in the vesicle lumen cannot reach the receptor of the lymphocyte membrane and therefore cannot trigger the stimulation Another possibility is the limited variability of the antigens in the insoluble fraction as contrasted with the soluble fraction

If EpSo and EpIn stimulated different lymphocyte populations one would expect that incubation of the lymphocytes with EpSo plus EpIn would give additive stimulation indices From the finding that stimulation of the lymphocytes with EpSo plus EpIn never exceeds the stimulation found by EpSo alone (Tables 3 and 4) we believe that the antigens of the EpIn fraction stimulate the same lymphocyte population as some or all antigens of the EpSo fraction This suggests that both epithelial fractions possess common antigenicity This is confirmed by the fact that EpSo-sensitised lymphocytes are also stimulated after incubation with the EpIn fraction (Table 5) The seemingly suppressive effect of EpIn on the SI of EpSo is caused by a suboptimal incubation period for EpSo of 4 days

The present study shows the importance of having correct conditions for the lymphocyte stimulation test These conditions have to be determined accurately before any conclusion from the outcome of the test can be made This is especially true for clinical application because the degree of stimulation may be very low The lack of optimal conditions when using human lymphocytes for detecting cellular immunity in ocular diseases may be one of the causes of the discrepancies found among the tests of various authors (Henley *et al* 1971, Shore *et al*, 1972, Henley and Okas, 1974, Szabo *et al* 1975, Freedman and Smit, 1974, Hammer, 1971, Hammer and Olah, 1975, Wong *et al*, 1971)

Another possibility for low or negative results in the lymphocyte stimulation test may be the type of antigen In this report it is shown that the insoluble epithelial fraction can elicit cellular immunity in rabbits Marak *et al* (1971) did not find stimulation of lymphocytes from patients with uveitis on incubation with sedimented antigens from several parts of the eye, nevertheless, they suggested that the major part of the active antigens was present in the insoluble fraction even though they were unable to demonstrate these antigens Antibodies to the HL-A antigens can be provoked in keratoplasty patients which demonstrates the antigenic properties of this insoluble part of the membrane (Stark *et al* 1973)

The lymphocyte stimulation test is believed to be an accurate test for detecting delayed-type hypersensitivity However we must be aware of the possible stimulation of B lymphocytes in addition to or instead of T-lymphocytes (Greaves *et al* 1974) From Tables 3 and 4 it appears that a high lymphocyte stimulation index and an intense skin reaction correlate with a low antibody titre The converse is also true This is in agreement with the results of Jevitz and Ekstedt (1971), who showed that, when cultured *in vitro* lymphocytes of rabbits favouring delayed hypersensitivity provoked a greater response to antigen than the B-lymphocytes It also appears that a high stimulation found with PHA-P correlated with a relatively high stimulation with EpSo and EpIn and a more intense skin reaction The transformation of lymphocytes by PHA is now well recognised as a measure of T-lymphocyte function this also points to a dominating stimulation of the T lymphocyte by EpSo and EpIn From this point of view one could imagine that the activity of the T-lymphocytes could be triggered by the soluble antigens This could be followed by graft rejection as a result of increasing cytotoxity of the T-lymphocytes by recognising the cross-reacting insoluble antigens

Table 5 *Stimulation indices of EpSo sensitised lymphocytes after incubation with EpSo and EpIn under optimal circumstances*

Rabbit no	SI	
	EpSo	EpIn
14	51.0	17.1
15	144.1	44.7
16	43.3	19.7

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CHAPTER 8

Ocular Antigens IX. Demonstration of Common Antigenicity in Corneal Epithelium and Other Tissues of the Bovine Eye

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The lymphocyte stimulation test was used as a sensitive probe for detecting common antigenicity Lymphocytes sensitized to corneal epithelium could be stimulated by the soluble antigens from corneal stroma lens epithelium iris retina and sclera Immunodiffusion revealed the presence of at least one common antigen in these tissues with the exception of corneal stroma and sclera Apart from this common antigen lens epithelium and iris shared one other antigen with corneal epithelium Between corneal stroma and corneal epithelium at least five common antigens could be detected

The involvement of various eye tissues in inflammatory eye diseases of different origin may be based on this common antigenicity

Key words immunology, lymphocyte stimulation, common antigenicity cornea, lens, retina, iris, sclera

1. Introduction

It has been assumed that accompanying disorders of the uvea are caused by the presence of common antigens in structures such as the lens the retina and the choroid (Rahi and Garner, 1976) Antigens of the uvea have indeed been detected in the retina (Perkins and Wood 1963) and in the corneal stroma and scleral stroma (Broekhuyse and Van der Eerden 1974) It was shown that patients suffering from diseases of the cornea may elicit delayed hypersensitivity to antigens from other ocular tissues which also points to the existence of common antigenicity (Shore Leopold and Henley 1972) The majority of the studies concerning common antigenicity between several eye tissues are based on immunodiffusion which is a relatively insensitive technique but is nevertheless suitable for a closer investigation into the identity of the antigens Serum proteins which hampered most of the investigations could not be detected in the corneal epithelium (Holt and Kinoshita, 1973, Hall, Smolin and Wilson, 1974) Moreover the epithelium is considered to be the most strongly antigenic part of the cornea (Berger, 1969) which makes this tissue very suitable for immunological studies of tissue specific antigens

The aim of the present study is to investigate the stimulation of lymphocytes sensitized to corneal epithelial antigens by antigens extracted from other tissues of the eye and to compare these results with those found by immunodiffusion

2. Materials and Methods

Isolation of the antigens

Bovine eyes were obtained from the slaughter house Immediately after death the eyes were enucleated, washed with saline, dripped with 0 25% chloramphenicol and transported on ice to the laboratory All further procedures were carried out at 0 4°C unless indicated otherwise Isolation of the soluble corneal epithelial antigens (EpSo) was carried out as described previously (Brinkman, Oerlemans-van Zutphen and Broekhuyse, 1978) After freeing the corneas from the residual epithelium they were removed and the endothelium

was scraped off The corneal stromas and the scleras were washed with saline and homogenized briefly in excess 0 01 M ammonium acetate at pH 7 2 using a mixer with rotating knife Homogenization was continued in an Ultra Turrax mixer in a nitrogen atmosphere After stirring the solutions for 30 min, the soluble stromal fraction (SaSo) and the soluble scleral fraction (ScSo) were isolated as a 450 000 × **g** min supernatant which was lyophilized Lens epithelium (with capsule), iris and retina were washed with saline and homogenized in 0 01 M ammonium acetate buffer at pH 7 2 in a Potter Elvehjem tube The solutions were stirred for 16 hr and the soluble antigen fractions (LeSo, IrSo and ReSo respectively) were isolated in the same way as SaSo and ScSo All preparations were stored at −20°C until used Determination of protein was carried out according to Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard The protein content on a dry weight basis amounted to 16% for ScSo and to about 50% for the other antigen preparations

Immunological procedures

New Zealand rabbits received 100 mg wet wt of bovine corneal epithelium in 1 ml phosphate buffered saline (PBS), emulsified with 1 ml Freund's complete adjuvant (FCA) subcutaneously at different places on the back Control rabbits were injected with FCA Isolation, culturing labelling and harvesting of the rabbit lymphocytes were carried out as described previously (Brinkman Oerlemans van Zutphen and Broekhuyse 1977) Stimulation of the lymphocytes was done with tissue antigens solubilized in Eagle's Minimal Essential Medium (MEM), 10 mg dry wt per ml and sterilized by sonication The degree of stimulation is reported as the stimulation index (SI) which is expressed as

$$SI = \frac{[^3H]\text{thymidine incorporation in stimulated cultures}}{[^3H]\text{thymidine incorporation in unstimulated cultures}}$$

$SI \geqslant 2$ is considered to be positive

Immunodiffusion was performed using 1% agarose in 0 1 M Tris Veronal buffer, pH 8 8 Immunoprecipitates were stained with Amido Black or Coomassie blue

3. Results

An optimal lymphocyte stimulation was obtained after 5 days cultivation with about 500 μg dry material from the stock preparation described above of EpSo, SaSo IrSo, ReSo and LeSo With the ScSo fraction a dose of 1000 μg dry material was needed for optimal stimulation

TABLE I

Stimulation of lymphocytes of rabbits sensitized with corneal epithelium and of FCA sensitized rabbits with soluble antigens extracted from several eye tissues and with bovine serum

Rabbit nos	EpSo	SaSo	LeSo	IrSo	ReSo	ScSo	BoS
1 9*	5 3 8 ± 2 3 9	1 9 8 ± 1 8 8	7 7 ± 8 0	2 1 1 ± 2 2 0	6 0 ± 4 8	5 1 5 0	1 4 ± 0 9
10–13†	0 9 ± 0 2	1 4 ± 0 3	1 6 ± 0 3	0 6 ± 0 1	1 0 ± 0 1	0 2 ± 0 1	1 5 ± 0 4
	$P = 0\,001$	$P = 0\,002$	$P = 0\,010$	$P = 0\,001$	$P = 0\,001$	$P = 0\,002$	$P = 0\,42$

Values represent average stimulation indices (SI) ± SD

P values refer to significance of differences between corneal epithelium sensitized rabbits (*) and FCA sensitized rabbits (†)

Under this regimen, the lymphocytes of nine rabbits sensitized with total corneal epithelium and of four FCA sensitized rabbits were incubated with the soluble antigens extracted from several other calf eye tissues. The results are summarized in Table I. Apart from EpSo, high lymphocyte stimulations were obtained with SaSo and IrSo. Positive stimulation after incubation with bovine serum (BoS) was noted in only one case. None of the control rabbits showed a positive lymphocyte stimulation upon incubation with the antigen mixtures used. The differences in S.I. between both groups of rabbits were statistically significant (Wilcoxon test) for all antigens with the exception of BoS ($P = 0{\cdot}42$).

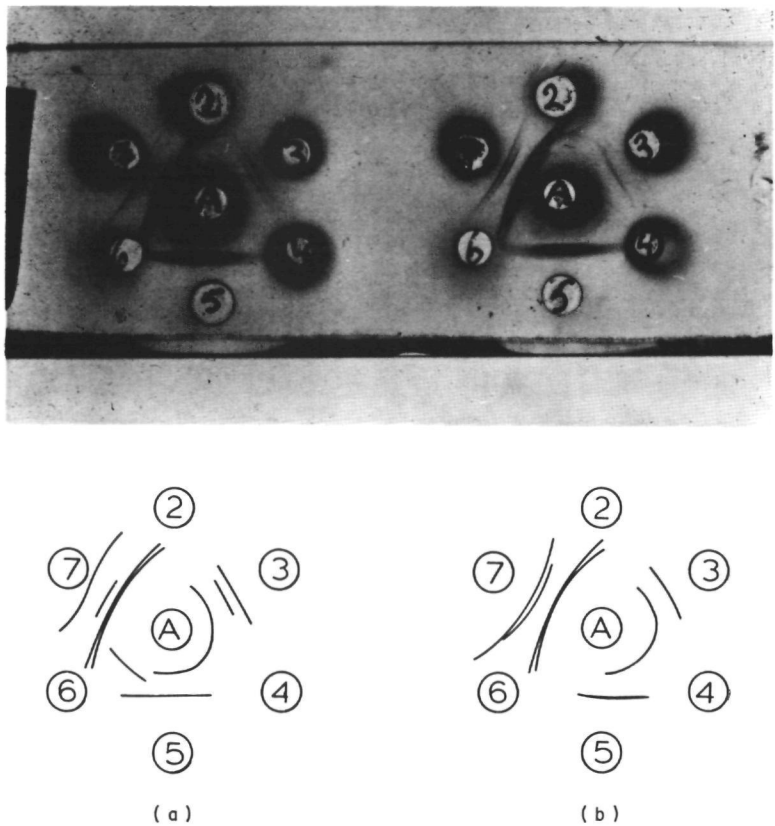

FIG. 1. (a) Reactions of anti-corneal epithelium antiserum (central well) prepared in rabbit with the soluble fractions of sclera (2), iris (3), retina (4), lens-epithelium (5), corneal stroma (7) and with bovine serum (6). (b) The same as a, however, the central well was filled with bovine serum (1 hr at 37°C) before applying the anti-corneal epithelium antiserum in order to absorb anti-serum protein antibodies.

For a closer investigation of the identity of the tested antigen fractions each antiserum to corneal epithelium was examined for precipitin line formation with the several soluble antigen fractions by immunodiffusion. Table II shows that each anti-corneal epithelium antiserum produced precipitin lines with antigens from other eye

tissues with the exception for the antigens extracted from the sclera Some of these lines showed reactions of identity The antigens extracted from corneal stroma, iris and lens epithelium gave more than one precipitin line In only one case a reaction could be detected between anti corneal epithelium antiserum and undiluted BoS (optimal concentration) (Table II and Fig 1) However, this reaction vanished by pre absorption of this anti-corneal epithelium antiserum with BoS [Fig 1(a, b)] as did one of the precipitin lines between anti corneal epithelium antiserum and iris The fusion between a precipitin line formed by lens epithelium, iris and retina soluble antigens [Fig 1(b)] reveals the presence of a common antigen (common antigenic determinant) in these tissues Besides this common antigen, iris and lens epithelium possess another antigen in common with corneal epithelium With the soluble antigen fraction of corneal stroma four precipitin lines could be detected using this particular antiserum [Fig 1(b)]

TABLE II

Immunodiffusion of anti-corneal epithelium antisera versus the soluble antigens extracted from several eye tissues and bovine serum

Antiserum used from rabbit no	Number of precipitin lines with						
	EpSo	SaSo	LeSo	IrSo	ReSo	ScSo	BoS
1	5[a]	4[a]	0	2[b]	1[b]	0	0
2	3	2	0	1	0	0	0
3	4[c]	3[c]	0	2[d]	1[d]	0	0
4	n d *	5	1	2	1	0	0
5	n d	3	1[e]	2[e]	1	0	0
6	n d	2	0	1	1	0	0
7	n d	5[f]	2	3[f]	1	0	1
8	n d	5[g]	1	1[g]	0	0	0
9	n d	5	0	2	1	0	0

Common antigenicity (a fused precipitin line) is indicated by corresponding superscript letters a g
* n d = not determined

4. Discussion

Cross reactivity between antigens extracted from retinal and uveal tissues and from lens epithelium was previously demonstrated by Nozaki Foster and Sery (1963) Also uvea, retina, corneal stroma and scleral stroma share antigenic determinants as was described by Perkins and Wood (1963) and by Broekhuyse and Van der Eerden (1971) Benezra (1976) showed that common antigenicity exists between soluble extracts from cornea, lens and retina using the lymphocyte stimulation test However, the influence of the serum proteins, present in retina and corneal stroma (Broekhuyse, 1972) was not investigated

The corneal epithelium is suitable for the study of the intrinsic antigens of ocular tissues because of the extreme low concentration or probable absence of serum proteins (Holt and Kinoshita, 1973, Hall, Smolin and Wilson, 1974) which is also shown in this report Berger (1971) prepared antisera against different antigens extracted from the bovine corneal epithelium and showed that apart from a tissue specific antigen, a common antigen could be detected in an extract of the iris as well

as in extracts of extra ocular tissues Rathbun, Johnson and Fusaro (1971) demonstrated that corneal epithelium and lens share antigens

The results of the present investigation reveal that antigens of the epithelium of calf cornea are also present in several other eye tissues Corneal stroma iris and lens epithelium share at least five, two and two antigens, respectively, with corneal epithelium Between anti corneal epithelium antiserum and soluble scleral antigens no precipitin lines could be demonstrated This is in agreement with the results of Whiteside, Hamada and Manski (1973) Still, the results of the lymphocyte stimulation test suggest cross reactivity between scleral and corneal epithelium antigens This discrepancy may be explained by the fact that the lymphocyte stimulation test is a parameter of T lymphocyte activity (Brinkman, Oerlemans van Zutphen and Broekhuyse, 1978) The immunodiffusion technique, however, determines the products of the B lymphocytes, i e immunoglobulins From this point of view it is possible that sensitization to the common antigens of the corneal epithelium and the soluble part of the sclera is predominantly T lymphocyte dependent A more sensitive detection of the immune response by the lymphocyte stimulation test is another possibility The absence of a positive stimulation of the lymphocytes of the rabbits injected only with FCA eliminates the possibility that common antigenicity was an expression of a cross-reactivity between ocular glycoproteins and bacterial proteins present in the adjuvant

We agree with the postulation of Whiteside, Hamada and Manski (1973) that besides ontogeny some other factor must play an active role in the antigenic nature of the several eye tissues, because the observed cross reactivity between the several eye tissues cannot be explained by common embryologic origin The corneal epithelium originates from the surface ectoderm as does the lens The retina and the pigment epithelium of the iris originate from the neural ectoderm, and the corneal stroma and the sclera, however from the mesoderm (Barber, 1955, Mann, 1949)

As has been discussed in various reports, it is likely that inflammatory diseases sometimes spread in the body as a result of immune reactions which are directed against cross reacting antigens in tissues other than the one primary involved Shore, Leopold and Henley (1972) revealed that patients suffering from corneal diseases can elicit delayed hypersensitivity to soluble antigens of iris, sclera and uvea Van der Eerden and Broekhuyse (1973) discussed ocular involvement in systemic immune disorders The present study confirms and extends previous investigations and shows clearly the extensive cross antigenicity between the various ocular tissues as revealed by using anti corneal epithelium antiserum Other antisera raised against serum free ocular tissue antigens may further extend our knowledge about this phenomenon and are currently in preparation in our laboratory

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CHAPTER 9

IMMUNOLOGICAL ACTIVITY TO DIFFERENT CORNEAL ANTIGENS IN PATIENTS WITH CORNEAL DISEASES

C.J.J. Brinkman, W.F. Treffers and R.M. Broekhuyse.

SUMMARY

Patients suffering from various corneal diseases and awaiting keratoplasty have been immunologically investigated in order to establish sensitisation to corneal antigens.

The presence of lymphocytes sensitised to the soluble fractions from human corneas, bovine corneal epithelium and bovine corneal stroma, which all possess common antigenicity, could be demonstrated in 30%, 50% and 23%, respectively, of all patients.

In none of these patients could a positive plasma antibody titre to human corneal antigens be detected. The results suggest the dominance of T-lymphocyte activity.

No correlation was found between the degree of corneal vascularisation and the presence of sensitised lymphocytes to human corneal antigens.

Arrangement of the patients according to diagnosis showed that especially those suffering from herpes simplex virus keratitis reacted positively to human corneal antigens. A possible explanation is given.

Lymphocytes of controls showed no or only very low stimulation with the soluble fractions of human corneas or bovine corneal stromas. The soluble fraction of bovine corneal epithelium stimulated the lymphocytes of 6 out of 19 controls.

The elimination of the donor corneal epithelium before transplantation may be beneficial in view of the involvement of histocompatibility antigens.

INTRODUCTION

Corneal grafts may be subject to immunological rejection. It is stated that an allograft reaction develops in about 12% of cases with good prognosis (17), which may extend to 65% in patients with preoperatively heavily vascularised corneas (8). The physiological state of the recipient cornea might influence the immune state of the patient.

The role of cellular immunity in corneal grafting has been demonstrated earlier in animal studies by passive transfer of sensitised lymphocytes (9) and by the detection of macrophage migration inhibition factor in the aqueous humour during corneal graft rejection (19). This factor has been shown to be an in-vitro correlate of cell-mediated immune reactions (5, 2).

In the present investigation the lymphocyte stimulation test has been used to estimate the state of presensitisation to corneal antigens in a group of patients awaiting keratoplasty. Furthermore this technique was used to compare the antigenicity of corneal stroma and epithelium to study a possible beneficial effect of removing the donor corneal epithelium before transplantation.

MATERIALS AND METHODS

Subjects- The investigation included 43 patients suffering from various corneal diseases for at least 6 weeks. All patients were evaluated for keratoplasty. Some patients received topical steroid therapy. Twenty-two healthy volunteers served as controls. Details are given with the results.

Antigens preparations- Human corneas, excised from fresh donor eyes, were stored at - 70° C until used. All further procedures were carried out at 0-4° C unless indicated otherwise. The corneas were cut in small pieces and homogenised in excess 0.01 M ammonium acetate buffer, pH 7.2, with an Ultra Turrax mixer in a nitrogen atmosphere. Homogenisation was continued by sonication, and the soluble corneal fraction was isolated by centrifugation for 30 minutes at 15000 g. The supernatant was lyophilised, and the product (HuCoSo) was stored at - 20° C until used. Protein content, based on dry weight, was determined according to Lowry et al. (10) and amounted to 50% when bovine serum albumin was used as standard. Corneal

epithelium soluble antigens (Bo-EpSo) were isolated from fresh bovine eyes as previously described (1). After removing epithelium and endothelium, the corneal stromas were treated for isolation of the soluble antigens (Bo-SaSo) as described above for Hu-CoSo. The protein content, based on dry weight, of Bo-EpSo and Bo-SaSo amounted to 65%.

Lymphocyte stimulation- A sample of heparinised blood was used for plasma preparation and the remainder was diluted with an equal volume of Eagle's Minimum Essential Medium (MEM; Gibco). The mononuclear cells were isolated from the diluted blood by Ficoll-Isopaque centrifugation (3). Lymphocytes, 3×10^5, were suspended in 1 ml MEM supplemented with 100 units of penicillin and 0.1 mg of streptomycin per ml and 20% heat-inactivated A-rh positive serum. Cultures of lymphocytes were incubated in triplicate with 1 mg Hu-CoSo, 1 mg Bo-EpSo or 0.5 mg Bo-SaSo (optimal antigen concentrations) for 6-7 days. All antigens had been solubilised in MEM to a concentration of 10 mg/ml and sterilised by sonication. Sterility was checked by incubating the antigens in culture medium. Because some patients were topically treated with corticosteroids (usually dexamethasone), the stimulation activity of the lymphocytes was controlled by incubation with 5 µg phytohaemagglutinin-P (Difco) for 3 days. Control cultures contained neither antigen nor mitogen. Twenty-four hours before harvesting 0.5 µCi tritiated thymidine (specific activity 25 curi/millimole) was added. Harvesting of the cells was done by filtration under reduced pressure through Millipore glass fibre filters. The filters were incubated in 0.5 ml Nuclear Chicago Solubiliser (NCS), diluted 1 to 3 with toluene based scintillation fluid containing 100 mg di-methyl-POPOP [2,2'-p-phenylen-bis-(5-phenyloxazol)] and 5 g PPO (2,5 - diphenyloxazol) per litre, for 30 minutes at 20° C. After addition of 9.5 ml scintillation fluid, containing 1 ml/l glacial acetic acid, the activity was counted in a liquid scintillation counter. The degree of stimulation is reported as the stimulation index (SI) which is expressed as: SI = mean counts per minute (c.p.m.) of cultures in the presence of antigen / mean c.p.m. of cultures in the absence of antigen.

An SI $\geq$ 2 with Hu-CoSo and an SI $\geq$ 3 with Bo-EpSo or Bo-SaSo was considered to be positive.

Antibody titre- The plasma antibody titre was estimated by the haemagglutination test (7). One ml 2% (V/V) tanned sheep erythrocytes

was incubated with 1 mg Hu-CoSo.

Immunodiffusion- Immunodiffusion was performed in 1% agarose in 0.1 M Tris-Veronal buffer, pH 8.8. Bo-EpSo and Bo-SaSo were solubilised in saline to a concentration of 10 mg/ml and 20 mg/ml respectively. In order to absorb antibodies to serum proteins the central well was filled with bovine serum and incubated for 1 hour at 37° C before applying the anti-total human cornea antiserum. This antiserum was prepared by injecting rabbits subcutaneously at multiple sites in the back with 5 mg human corneal antigens (protein based) solubilised in 1 ml phosphate buffered saline (PBS), pH 7.4, and emulsified with 1 ml Freund's complete adjuvant. Boosting was performed 4 and 6 weeks later with 5 mg human corneal antigens solubilised in 1 ml PBS. One week after the last injection the rabbits were bled.

RESULTS

Lymphocyte stimulation- A stimulation index of 10 or more was found after incubating the lymphocytes of patients and controls with PHA-P. This suggests that, despite the fact that some patients received corticosteroid therapy, their lymphocytes still possessed the potentiality to be stimulated. The stimulation activity of the different corneal antigen fractions on the lymphocytes of the patients with corneal disease and the healthy persons is shown in Fig. 1. A significant difference (Mann and Whitney U-test) was found between the stimulation indices of the patients with corneal disease and the healthy subjects after incubation of their lymphocytes with human corneal antigens ($P < 0.002$). No significant difference could be observed between both groups of subjects when tested with bovine corneal stroma antigens or bovine corneal epithelium antigens ($P > 0.09$, for both antigen fractions). The relation between the diagnosis of the patients and the presence of sensitised lymphocytes is also demonstrated in Fig. 1. In comparison with control subjects patients suffering from keratitis caused by herpes simplex virus (HSV) infection showed a statistically highly significant difference in stimulation activity after incubation of their lymphocytes with human corneal antigens ($P < 0.001$). No difference was found with bovine corneal epithelium antigens ($P > 0.06$) and bovine corneal stroma antigens ($P > 0.06$). The lymphocytes of patients suffering from bul-

lous keratopathy reacted also clearly positively with human corneal antigens ($P < 0.005$).

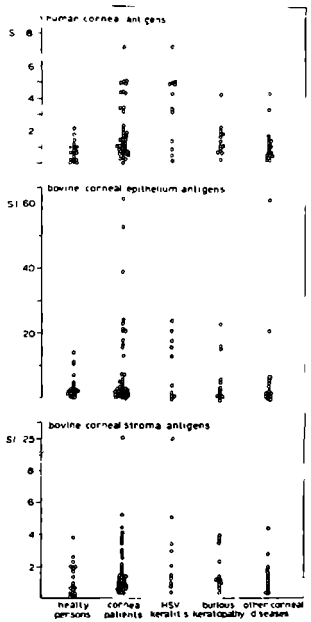

Fig. 1. *Stimulation of lymphocytes of healthy subjects and patients with corneal disease with human corneal antigens, bovine corneal epithelium antigens, and bovine corneal stroma antigens expressed as stimulation index (SI). The results of the patients suffering from HSV keratitis, bullous keratopathy, and other corneal diseases have been taken together in the group indicated as cornea patients.*

With bovine corneal epithelium and bovine corneal stroma antigens no statistically significant difference between these patients and healthy subjects could be detected ($P > 0.1$, for both antigen fractions). Patients suffering from keratoconus (1), scrofulosa (1), herpes zoster virus keratitis (1), Fuchs's dystrophy (1), pterygium (1), Peters's anomaly (2), physically or chemically induced keratitis (7) or idiopathic stromal keratitis (3) (classification according to Moore and Aronson (14)) showed no statistically significant difference in lymphocyte activation with either antigen as compared to controls ($P > 0.05$ for each antigen).

An $SI \geq 2$ with human corneal antigens and an $SI \geq 3$ with bovine corneal antigens being considered a positive lymphocyte response, lymphocytes sensitised to the soluble fractions from human corneas, bovine corneal epithelium, and bovine corneal stroma could be demonstrated in 30%, 50% and 23%, respectively, of all patients. According to this criterion significant differences (Fisher's one-sided exact test) in lymphocyte activity with human corneal anti-

gens ($P < 0.0005$) and bovine corneal stroma antigens ($P < 0.05$) were found only between patients suffering from herpes simplex virus keratitis and healthy persons, as shown in Table I. Six control subjects (31%) reacted positively with bovine corneal epithelium antigens.

Table I. *Stimulation of lymphocytes of patients, suffering from corneal diseases and of healthy persons by Hu-CoSo, Bo-EpSo and Bo-SaSo.*

Diagnosis	Number of total Subjects (with Percentage) with Positive SI to		
	Hu-CoSo	Bo-EpSo	Bo-SaSo
Bullous keratopathy	3/14 (21%)	7/14 (50%)	4/14 (29%)
Herpes simplex virus keratitis	8/12 (67%)*	7/11 (64%)	4/10 (40%)*
Other corneal diseases**	2/17 (12%)	6/15 (40%)	1/15 (7%)
Healthy***	1/22 (5%)	6/19 (32%)	1/18 (6%)

* Significant at $P < 0.05$ (Fisher's one-sided exact test).

*** 18 healthy subjects were tested with Bo-SaSo, 19 with Bo-EpSo and 22 with Hu-CoSo.

** See text (Results).

Corneal vascularisation might influence the state of immunity of patients. However, as shown in Table II, no relation could be detected between the degree of corneal vascularisation and the presence of lymphocytes sensitised (i.e., SI ≥ 2) to human corneal antigens. Of 25 patients with heavily or mildly vascularised corneas 6 (24%) reacted positively with human corneal antigens. The patients with avascular corneas reacted in 7 (39%) of the 18 cases positively with this antigen fraction.

Antibody titre- The plasma antibody titre to human corneal antigens of all patients and controls was zero.

Immunodiffusion- Fig. 2 demonstrates the formation of 1 precipitin line between rabbit anti-human cornea antiserum and Bo-EpSo and 2 precipitin lines between rabbit anti-human cornea antiserum and Bo-SaSo. With bovine serum no precipitin line was found.

Table II. *Relation between corneal vascularisation and lymphocyte stimulation with Hu-CoSo.*

Degree of vascularisation	Stimulation Index	
	Positive	Negative
Heavy	4/13 (31)[x]	9/13 (69)
Mild	2/12 (17)	10/12 (83)
No	7/18 (39)	11/18 (61)

[x] Number of total patients (with percentage).

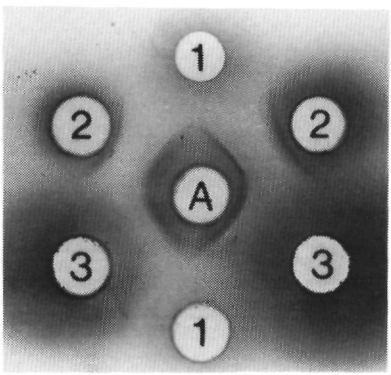

Fig. 2. *Immunodiffusion of anti-human cornea antiserum (A) versus bovine serum (1), bovine corneal stroma antigens (2) and bovine corneal epithelium antigens (3). The central well was pre-filled with bovine serum for 1 hour at 37° C in order to absorb anti-serum protein antibodies.*

DISCUSSION

Sensitisation of animals by tissue transplantation or antigen injection followed by keratoplasty leads to enhancement of the rejection of the corneal graft (11, 15, 21). Corneal graft rejection may partly be related to cell-mediated immunity (16, 9, 19), which may be detected by the lymphocyte stimulation test (4). Our results are consistent with this hypothesis by showing the presence of corneal antigen-sensitised lymphocytes in the blood of patients suffering from corneal diseases. The observed difference in lymphocyte responses due to different corneal antigens suggests some kind of antigen specificity of this immune activity.

The use of bovine corneal epithelium antigens and bovine corneal stroma antigens for investigation of a possible sensitisation of human beings to these antigens has been partly justified by immunodiffusion. Clear precipitin lines could be detected between anti-human cornea antiserum and the soluble antigens of these bovine tissues, indicating the presence of common antigenic determinants. The relatively high percentage of the controls reacting positively with the soluble antigens from bovine corneal epithelium as compared to stroma is a drawback and may be due to histoincompatibility. Incubation of human lymphocytes with these antigens may lead to a stimulation by histocompatibility antigens (or soluble precursors), probably more being present in the soluble fraction of the epithelium than in the soluble fraction of the stroma. The results described in this report may therefore suggest that separation of the epithelium from the donor cornea before keratoplasty eliminates most of the histocompatibility antigens and consequently might increase the chance of success.

Arrangement of the patients into groups according to the diagnosis of the corneal disease showed that bullous keratopathy sometimes caused an increased lymphocyte stimulation. The oedematous state of the cornea might result in a loss and alteration of corneal antigens in these patients, which might explain this increased response to human corneal antigens as compared to that of controls. Further evaluation of the results shows that especially those patients suffering from herpes simplex virus keratitis reacted positively with human corneal antigens. This group of patients appeared also to be more sensitised to bovine corneal epithelium and bovine corneal stroma antigens than the other groups of patients (Fig. 1). For bovine corneal epithelium antigens, however, this difference was statistically significant only at the $P = 0.1$ level.

Several explanations are possible for the high number of herpes simplex virus keratitis patients showing lymphocyte stimulation. The intense vascularisation of the cornea often encountered in this type of patients might create the possibility of lymphocytes coming into contact with corneal antigens. However, the influence of another or additional factor is indicated by the absence of a correlation between corneal vascularisation and a positive lymphocyte response (Table II). This is supported by the fact that corneal vascularisation is also seen in other forms of keratitis without eliciting an immune

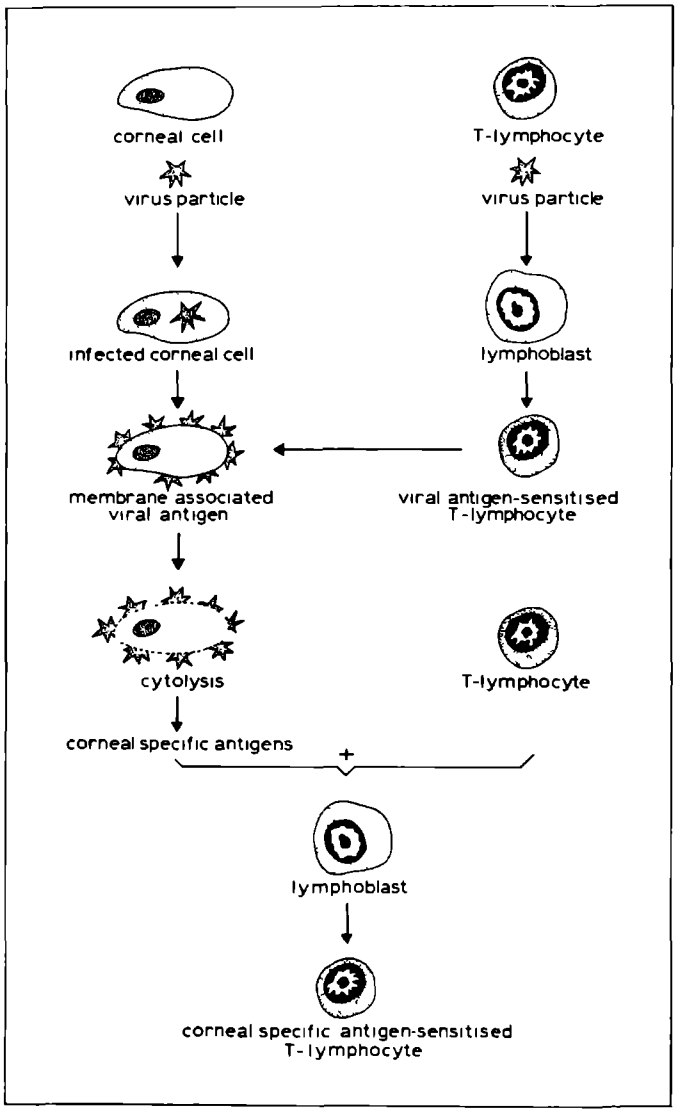

Fig. 3. *Corneal cells exposing membrane associated viral antigens are recognised by peripheral viral antigen-sensitised lymphocytes. The consequent cytolysis results in release of corneal specific antigens which activate the immune system of the patient. This is measured by the in-vitro lymphocyte stimulation.*

response. It is likely that the change of cell-surface antigens of corneal cells by incorporation of viral antigens, demonstrated after infection of the cornea with the herpes simplex virus (6, 12), might be one of the primary causes of the immune response. Cell-mediated immune activity to viral antigens has been demonstrated after herpes simplex virus infection in animals (20, 13) and in man (18). A second contact between systemic sensitised lymphocytes and viral antigens present on the corneal cell surfaces may lead to corneal opacification, probably by destruction of the corneal cells by direct contact or by the release of cytolytic lymphokines. The consequent liberation of soluble corneal specific antigens together with high vascularisation might explain the large number of patients with herpes simplex virus keratitis who are sensitive to corneal antigens, resulting in poor prognosis for successful keratoplasty. This hypothesis is illustrated in Fig. 3. The absence of detectable plasma antibodies to human corneal soluble antigens suggests also in this particular case that especially T-lymphocytes are responsible for this immune activity.

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According to Zinn and Mockel-Pohl (24), lens proteins are considered foreign material with regard to the reticuloendothelial system. These antigenic properties are probably the result of the isolation of the lens proteins by the lens capsule that is already visible as early as 45 days following fertilisation (19). Uhlenhuth (20) was one of the first to demonstrate the antigenicity of bovine lens crystallins after injection of these proteins into rabbits. Verhoeff and Lemoine (21) postulated an autoimmunity to lens proteins in patients suffering from phacogenic uveitis.

The mammalian lens crystallins which make up the largest part of the soluble protein fraction of the lens can be distinguished mainly by their differences in electrophoretic mobility and molecular weight in three fractions i.e. alpha-, beta- and gamma crystallins. It is generally accepted that alpha crystallin, which has the highest molecular weight, is the most antigenic of the soluble lens fraction (8, 12) and consequently might be responsible for the induction of different forms of inflammatory diseases after contact with the immune system. This may especially be important with respect to the presence of antigenic determinants resembling alpha crystallin in other parts of the eye (11, 8, 10, 5).

The induction of an immune response in laboratory animals by injecting autologous lens proteins was only incidentally successful (9, 17). The failure of other attempts may partially be explained by the presence of the earlier mentioned lens protein-like determinants in both other ocular tissues and in extra-ocular tissues (18) resulting probably in a state of immunologic tolerance to these proteins. Nevertheless, it was demonstrated by a number of investigators that patients suffering from lens-induced uveitis showed a delayed-type hypersensitivity reaction after intradermal injection of lens proteins (21, 23). These results were confirmed

by Hammer and Olah (6) and Kincses and Török (7). They used lymphocyte transformation and leukocyte migration inhibition as in vitro parameters to reveal the presence of a state of autoimmunisation to purified alpha crystallin and total lens crystallins in patients suffering from phacogenic uveitis and after complicated cataract extraction.

Wirostko and Spalter (22) reported on the development of anti-lens antibodies in the serum of patients suffering from endophthalmitis phacoanaphylactica(a synonym of phacogenic uveitis). Most of these patients with anti-lens antibodies had a significant amount of lens material in the anterior chamber, while no anti-lens antibodies were detectable at the time of operation. This study clearly suggests the involvement of lens remnants in the induction of a humoral autoimmune response which might be responsible for the development of phacogenic uveitis. Patients who underwent intracapsular lens extraction failed to demonstrate anti-lens antibodies probably due to the lack of lens remnants in the aqueous humor. The involvement of anti-lens antibodies in phacogenic uveitis has been investigated more exactly by Marak and his colleagues. Injury to lenses of rats, which have been previously sensitised with homologous lens proteins, resulted in inflammation known as experimental lens-induced granulomatous endophthalmitis (ELGE), resembling, from the histopathological point of view, human lens-induced uveitis (13). The iris and ciliary body of the rat eyes were hyperemic with an infiltration of small lymphocytes and histiocytes in the stroma of these tissues. Plasma cells and eosinophils were also detectable. The possibility to induce ELGE after passive transfer of immune serum immediately followed by injury of the lens (14) suggest an active role of anti-lens antibodies in this disease. Indeed, anti-lens antibodies and complement were bound to the injured lenses of rats previously immunised with lens proteins (15).

A subject for discussion is the discrepancy between the difficulty in inducing autosensitisation in animals by injecting autologous lens material and the often encountered state of autosensitisation in patients suffering from phacogenic uveitis. One of the possible explanations is that in contrast to experimental studies most patients with phacogenic uveitis suffered from a cataractous

lens which was removed by extracapsular lens extraction. Both the cataractous lens as well as the extensive manipulation within the eye during extraction may be responsible for the state of immunisation in these patients. The cataractous human lenses usually contain a high content of insoluble albuminoid (12). In animals, albuminoid was demonstrated to be more stimulatory for the reticuloendothelial system as compared to soluble lens proteins (1, 2, 12). Liberation of this highly antigenic material into the aqueous humor during extracapsular lens extraction may result in stimulation of the immune system of the patient. The study of Wirostko and Spalter (22) supports this hypothesis. However, intravitreal injection of albuminoid into rats produced a spontaneous uveitis with the characteristics of a cellular immune response not earlier than after 6 days (2). The fact that in patients phacogenic uveitis can be detected sometimes 1 or 2 days following lens extraction suggests the immune system to be activated earlier i.e. before operation. This hypothesis could recently be confirmed by the detection of circulating lymphocytes sensitised to lenticular antigens in patients with cataractous lenses who had not been operated on before (3). Furthermore, it was suggested that the autoimmune response in these patients had been developed by small lenticular lens antigens presumably of the beta- and gamma crystallin fraction or by fragments of other crystallins formed by proteolysis and/or ageing processes. This theory has been partially confirmed by the investigation of Marak et al. (16) who were able to demonstrate the equally immunopathogenic nature of alpha-, beta- and gamma crystallins in their ability to develop ELGE in rats. Our subsequent studie on this topic showed that autosensitisation by lens antigens may have occurred even before any lens opacity was visible (4) and consequently, that autoimmune phenomena may be involved in the pathogenesis or progression of some types of cataracts.

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CHAPTER 10

Cell mediated immunity in relation to cataract and cataract surgery

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SUMMARY Two groups of patients suffering from cataract were investigated by the lymphocyte stimulation test One group was tested before and the other after cataract surgery Human (total) lens crystallins and bovine alpha crystallin were used as the antigens Lymphocytes of healthy persons showed practically no positive stimulation with either antigen Of the patients tested before operation 38% reacted positively with lens crystallins and 15% with alpha crystallin This difference in sensitisation suggests a leakage of lenticular antigens other than alpha crystallin out of the lens during cataract progression Extracapsular lens extraction caused an increase in the number of patients reacting positively with lens crystallins and alpha crystallin as compared to patients not operated upon Intracapsular lens extraction on the other hand resulted in less sensitisation to lens crystallins as compared to patients not operated upon

The isolation of the lens proteins from the fetal circulation early in embryonic life, the lack of innervation, and the complete avascularity of the adult lens might suggest that the lens proteins initiate an autologous sensitisation after entering the aqueous humour However, it has only recently been possible to detect antibodies to homologous lens proteins in small amounts in rabbits (Misra *et al*, 1977) Earlier attempts to stimulate anti-lens antibody formation were successful only by sensitisation with homologous lens proteins in combination with an adjuvant (Halbert *et al*, 1965) The presence of an adjuvant still seems necessary for the immune response to autologous lens antigens (Rahi *et al*, 1977)

Phacogenic uveitis or lens-induced uveitis, which is characterised by a sterile iridocyclitis, is thought to be the result of autosensitisation to lens proteins Clinically this inflammation may develop within 2 days after perforation of the lens capsule (Schlaegel, 1975), indicating a secondary immune response The primary response of the immune system could have been evoked by leakage of lenticular antigen into the aqueous humour during cataract maturation Because it is difficult to induce a good antibody response after immunisation with homologous or autologous lens proteins it is likely that cellular immunity plays a role in the induction of phacogenic uveitis

The present study deals with the *in-vitro* stimulation by lens crystallins and alpha crystallin of lymphocytes of patients suffering from cataract in order to investigate this possible leakage In addition, a comparison of lymphocyte activation has been made before and after cataract surgery

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Patients and methods

The investigation covered 49 patients suffering from cataract, who were divided into 2 groups The first group consisted of 21 patients who were tested 1 day before cataract surgery and who had not been operated on before The second group consisted of 28 patients who were tested at least 18 days after cataract surgery Patients who received corticosteroid or other anti-inflammatory preparations during the investigation were excluded Twenty-two healthy volunteers (age range 20 to 64 years, including 8 persons between 51 and 64 years) served as controls Two of them could not be tested with alpha crystallin

PREPARATION OF ANTIGENS

Cataractous lenses (age range of patients 60 to 70 years) removed after intracapsular lens extraction were used after storage at −70°C Lens crystallins were isolated by homogenisation of the decapsulated lenses in excess 0 01 M ammonium acetate buffer (pH 7 2) in a Potter Elvehjem tube at 4 C The

suspension was centrifuged for 1 hour at 15 000 *g* at 4 C and the supernatant was lyophilised and stored at −20 C until used Protein content of the dry w ght material (lens crystallins) was determined by the Lowry method (Lowry *et al*, 1951) and amounted to 80% Serum albumin was used as standard

Alpha crystallin, the non-species-specific lens crystallin (Halbert *et al* 1965), was isolated from fresh decapsulated bovine lenses as follows Bovine lens crystallins were isolated as from human lenses, 80 mg of bovine lens crystallins were dissolved in 5 ml 0 1 M ammonium acetate buffer (pH 8 0) supplemented with 0 5% butanol and applied to a Sepharose 6B column (100 × 2 5 mm) (Bours and Brahma, 1973) The column was eluted with the same buffer The fractions containing pure alpha crystallin (as checked by isoelectrofocusing) (Bours and Brahma, 1973) were lyophilised and stored at −20 C until used

LYMPHOCYTE STIMULATION ASSAY (LSA)

Heparinised blood was diluted with an equal volume of Eagle s minimum essential medium (MEM) The mononuclear cells were isolated from the diluted blood by Ficoll-Isopaque centrifugation (Du Bois *et al*, 1973) Lymphocytes, 3×10^5, were suspended in 1 ml MEM supplemented with 100 units of penicillin and 0 1 mg of streptomycin per millilitre and 20% heat-inactivated A rh positive serum Cultures of lymphocytes were incubated in triplicate with 1 mg human lens crystallins or 50 µg bovine alpha crystallin for 6 to 7 days Viability and stimulation activity was checked by incubating the lymphocytes with 5 µg phytohaemagglutinin P (Difco) for 3 days Control cultures contained neither antigen nor mitogen Sterility was controlled by incubating the antigens alone in the culture medium Twenty-four hours before harvesting, 0 5 µCi tritiated thymidine (specific activity 25 curi/millimole) was added Harvesting of the cells was done by filtration under reduced pressure through Millipore glassfibre filters The filters were incubated in 0 5-ml Nuclear Chicago Solubilizer (NCS), diluted 1 to 3 with toluene based scintillation fluid containing 100 mg dimethyl-POPOP* and 5 g PPO† litre for 30 minutes at 20 C After addition of 9 5 ml scintillation fluid containing 1 ml glacial acetic acid per litre the activity was determined in a liquid scintillation counter The degree of stimulation was expressed as the stimulation index (SI) SI counts per minute (cpm) in the presence of antigen divided by cpm in the absence of antigen An SI > 3 with human lens crystallins and an SI > 2 with bovine alpha crystallin was considered to be positive

*POPOP 2 2 *p* phenylen bis (5 phenyloxazol)
†PPO 2 5 diphenyloxazol

Results

LSA BEFORE CATARACT SURGERY

Lymphocytes from 21 patients, obtained 1 day before cataract surgery, were cultured with total lens crystallins and alpha crystallin Eight (38%) of these patients showed a positive lymphocyte response with total lens crystallins (Table 1, Fig 1) Two healthy persons (9%) showed a positive lymphocyte stimulation with lens crystallins (persons aged 34 and 64 years) (Fig 1) The difference in total lens crystallins sensitisation between healthy persons and cataract patients was statistically significant ($P < 0.05$, Fisher s one-sided exact test) Alpha crystallin produced a positive lymphocyte culture in 3 (15%) of the 20 patients investigated (Table 1, Fig 1), which was statistically insignificant in comparison with the group of 20 healthy persons, who did not react with alpha crystallin ($P > 0.1$) (Fig 1) In addition to 5 patients with a mature cataract 14 patients suffered from a cataract in which the lens cortex was involved, and 8 patients

Table 1 *Stimulation of lymphocytes of patients suffering from cataract before cataract surgery*

Case	Sex	Age (yr)	Cataract type			Stimulation index with	
			Nuclear	Cortical	Other	Lens crystallins*	Alpha crystallin†
1	M	77				–	–
2	M	58			Mature	–	–
3	M	63	–			–	–
4	F	39			Mature	–	–
5	M	38					–
6	M	42				–	–
7	F	78	–	–		–	
8	M	75				–	–
9	M	57			Mature	–	–
10	M	66				–	–
11	M	66				–	–
12	F	71				–	–
13	M	64				–	–
14	M	53					–
15	M	64			Mature		
16	F	71			Mature		–
17	F	76	–			–	–
18	M	57				–	–
19	M	79	+	+		–	–
20	M	67		–		–	–
21	F	83	+			–	ND‡

* SI 3 5 + SI 5 7 + + − SI > 7
\+ SI 2 4 − SI 4–6
‡ Not determined

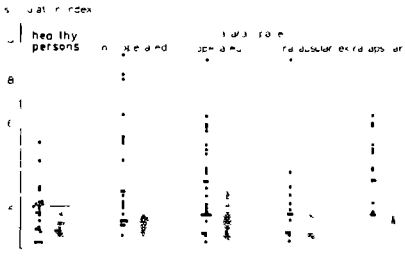

Fig 1 *Stimulation of lymphocytes of healthy persons and cataract patients by total human lens crystallins (●) and bovine alpha crystallin (○) The influence of cataract surgery and type of lens extraction (intracapsular and extracapsular) are shown An SI ≥ 3 with total human lens crystallins and an SI ≥ 2 with bovine alpha crystallin (horizontal bars) was considered to be positive*

Table 2 *Stimulation of lymphocytes of patients after cataract surgery*

			Stimulation index with		Lens extraction	
Case	*Sex*	*Age (yr)*	*Lens crystallins**	*Alpha crystallin†*	*Intra-capsular*	*Extra-capsular*
22	F	69	−	−	−	
23	M	28	+	+		+
24	M	39	−	−		+
25	F	69	−	−	−	
26	M	58	−	−	−	
27	F	63	−	−		−
28	M	78	−	−		
29	M	77	−	−		−
30	F	60	−	−		−
31	M	71	−	−		−
32	F	66	−	−	+	
33	F	65	−	−		−
34	M	73	−	−		−
35	F	86	−	−	+	
36	F	65	+ +	−		−
37	M	42	+ +	+		−
38	M	67	−	−	−	
39	M	66	+	+ +		+
40	F	78	+ +	+ + +		
41	F	80	+	+	+	
42	F	71	−	−		
43	M	66	+	−		
44	M	62	+ +			−
45	M	64	−	−		
46	M	82	−	−	−	
47	F	83	−	−	−	
48	F	71	− + +	ND‡	−	
49	M	57	−	−		

* + = SI 3–5, + + = SI 5–7 − − SI > 7
† + = SI 2–4, + + = SI 4–6, + + − = SI > 6
‡ Not determined

suffered from a cataract in which the lens nucleus was involved

LSA AFTER CATARACT SURGERY

A positive stimulation index with total lens crystallins was found in 13 (46%) of the 28 patients who underwent cataract surgery (Table 2, Fig 1), which is about the same percentage as before cataract surgery Lens extraction produced a rise in the number of patients who reacted positively with alpha crystallin (Table 2, Fig 1) Seven patients (26%) of the 27 were alpha crystallin sensitive after operation, which was significantly higher than in the group of healthy persons ($P < 0.05$)

Table 2 and Fig 1 also show the influence of the type of cataract extraction on the sensitisation to total lens crystallins and alpha crystallin Of 14 patients who underwent intracapsular lens extraction 3 patients (21%) reacted positively with lens crystallins and 2 (15%) of the 13 tested patients reacted positively with alpha crystallin These numbers were not significantly different from those of healthy persons ($P > 0.1$ for both antigen fractions) However, of 14 patients who underwent extracapsular lens extraction 10 (71%) showed sensitisation to lens crystallins and 5 (35%) to alpha crystallin This difference in alpha crystallin and lens crystallins sensitisation between patients who underwent extracapsular lens extraction and healthy persons was statistically significant ($P < 0.01$ and $P < 0.0005$, respectively)

Discussion

Differences have been found in protein content as well as in protein composition between normal and cataractous lenses Declining lens transparency appears to be associated with a decrease in the quantity of soluble lens proteins and an increase in the amount of insoluble proteins (Mach, 1963) A preferential decrease in low molecular weight proteins during progressive cataract formation was found by Sephadex gel filtration (François *et al*, 1965) Several authors suggested that these proteins are gamma crystallin (Maisel and Goodman, 1965, Croft, 1973) According to Harding and Dilley (1976) different explanations are possible for this decline in low molecular weight proteins in the lens, namely, by leakage through the lens capsule, by conversion into insoluble proteins, by cross-linking, or by a lack of synthesis Mach (1963) has already demonstrated that the increase in protein insolubilisation cannot account for the decrease in quantity of soluble low molecular weight lens proteins during cataractogenesis Charlton and van Heyningen (1968) considered the possibility of leakage of low

molecular weight proteins out of the lens but their results did not lead to definite conclusions

If leakage of lenticular proteins does occur during opacification of the lens, an immune response could be provoked leading to a population of sensitised lymphocytes in the patients' blood The present investigation supports this by showing that about 38% of the cataract patients (who had not been operated on before) had lymphocytes which could be stimulated by lens crystallins (Table 1) Normal persons showed practically no positive reactions with lens crystallins Only 3 (15%) of the 20 patients investigated reacted positively with alpha crystallin (Table 1) One of these 3 patients suffered from a mature cataract and another patient suffered besides from cataract from recurrent uveitis None of the control persons showed a positive response to alpha crystallin As is shown in Table 1, most (66%) of the patients who had not been operated on before suffered from a cortical cataract in contrast to 38% of these patients with a nuclear cataract

Philipson (1973) compared the morphological changes in both cortical and nuclear cataractous lenses by electron microscopy, microradiography, and light scattering The cortical opacities appeared to be accompanied by an enlargement of intercellular spaces followed by lens membrane damage The human nuclear cataract looked completely different In this type of cataract lens fibre cells and membranes did not show any difference from those of normal lenses The morphological changes commonly seen in cortical cataractous lenses may lead to selective permeability of the lens capsule, resulting in liberation of low molecular weight lens proteins, subsequently provoking an immunological response The high percentage of patients with cortical cataract, present in the group of patients who had not been operated on before, strengthens the idea that leakage of the lenticular proteins depends on the condition of the lens cortex rather than the lens nucleus

Cataract surgery seems to have little influence on the sensitisation of the lymphocytes to total lens crystallins On the contrary, after cataract surgery an increase in the number of patients sensitive to alpha crystallin could be detected

Separating the patients according to the type of cataract extraction showed that after extracapsular lens extraction 71% of the patients reacted positively with lens crystallins and 35% with alpha crystallin Of patients who underwent intracapsular lens extraction, however, 21% showed a sensitisation to lens crystallins and 15% to alpha crystallin This difference in sensitisation to lens crystallins and to alpha crystallin between both types of extraction procedures can be explained by the liberation of lens antigens into the aqueous humour, which is accomplished during extracapsular lens extraction Intracapsular lens extraction results in a complete removal of the source of lens antigens The latter also explains the lower percentage of patients showing sensitivity to lens crystallins after intracapsular lens extraction as compared to the patients not operated on

The results suggest that lenticular proteins other than alpha crystallin are free to pass the lens capsule predominantly in cortical cataractous lenses After entering the aqueous humour a primary immune response can be induced leading to a population of sensitised lymphocytes This primary sensitisation possibly concerns a cell mediated response because (*a*) Attempts to induce anti-lens antibodies were successful only in the presence of an adjuvant (Halbert *et al* 1965), (*b*) low molecular weight lens proteins tend to aggregate and precipitate after leakage from the lens (Charlton and van Heyningen, 1968), a phenomenon which favours T-cell activation (Manski, 1973)

Phacogenic uveitis is a sterile inflammation and is supposed to be the result of an immunological reaction to lens remnants present in the aqueous humour, especially after extracapsular lens extraction This type of uveitis develops sometimes very soon (1 or 2 days) after lens extraction (Schlaegel, 1975), which made us conclude that we are dealing with a secondary immune response, primary sensitisation being evoked by low molecular weight lens crystallins

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CHAPTER 11

LENS PERMEABILITY CHANGES IN RELATION TO X-RAY INDUCED CATARACT AS DETECTED BY CELL-MEDIATED IMMUNE ACTIVITY AND AQUEOUS HUMOR COMPOSITION

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SUMMARY

The experimental radiation cataract in rabbits served as a research model in an investigation of the relation between cataract progression and lens permeability changes. Cataract progression was followed by slit-lamp examination while lens permeability changes were detected by the activation of the immune system of the rabbits by lens proteins, and comparison of the composition of the aqueous humor of both eyes.

Irradiation of the eye lens resulted in an increase of the calcium concentration of the surrounding aqueous humor 4 weeks later whereas no changes of the sodium and potassium concentration were detected. Sensitisation of the cellular immune system of the rabbits as detected by skin tests and lymphocyte stimulation was found to be maximal 8 weeks following X-ray treatment. This sensitisation was predominantly directed to low molecular weight lens proteins, i.e. proteins other than intact alpha crystallin. Lens opacities could be detected not earlier than 12 weeks following X-irradiation.

The results point to leakage of low molecular weight lens proteins out of the lens, resulting in autoimmunisation, before cataract is clinically detectable.

INTRODUCTION

Senile cataract is one of the most frequently encountered diseases of the eye. Its pathogenesis, however, is still a matter of

speculation. As demonstrated in a previous investigation, lymphocytes of patients suffering from senile cataract could be stimulated by human lenticular antigens (2). Cortical opacities seemed to be an important prerequisite for this autoimmune process, which might be induced by leakage of lenticular antigens. Earlier, Sandberg (12) found increased amounts of alpha crystallin in the aqueous humor of patients suffering from senile cataract as compared to the aqueous humor in contact with clear lenses.

The question whether leakage of proteins from human lens precedes the clinical signs of lens opacity or the reverse is hardly to answer. The alterations in lens metabolism and protein distribution observed after X-irradiation show some similarities with those induced by ageing (6, 8).

In the present study we have used the radiation cataract as a model for the investigation of protein leakage out of the lens. After irradiation of one eye lens of young rabbits the composition of the aqueous humor of both eyes was compared and the immune response to homologous lenticular antigens was investigated by lymphocyte stimulation and skin tests at different time intervals. Cataract progression was followed by slit-lamp examination.

MATERIALS AND METHODS

The experiment was performed twice as described below.

Irradiation- Irradiation of the right lenses of 20 New Zealand rabbits (age 8 weeks) was carried out with a Stabilipan Siemens 77. The rabbits were anaesthetised by Nembutal. Each eye received a dose of 1800 Rad in a 23 min period. The radiation distance was 37 cm and radiation field was 2 cm. A Thoreus II filter was used. Slit-lamp examination was performed every 2 weeks. Two days, and 4, 8, 12 and 20 weeks after irradiation 4 rabbits were killed.

Antigen preparation- Rabbit lenses were homogenised in 0.01 M ammonium acetate buffer, pH 7.2, at 4° C. The soluble fraction was isolated by centrifugation at 15000 x g for 30 min at 4° C and lyophilisation of the supernatant. The product (Ra-LeSo, soluble rabbit lens proteins) was stored at -20° C. Alpha crystallin was isolated from Ra-LeSo by Sepharose 6B column gel filtration (1) using 0.1 M ammonium acetate buffer, pH 8.0 (containing 0.5% butanol) as elution

fluid. The alpha crystallin peak was checked for purity by isoelectric focusing (1). One band was observed (at pH 4.9). The lyophilised product (Ra-α) was stored at -20° C.

Skin tests- Forty-eight hours before killing, each rabbit was injected intradermally with 100 µg Ra-LeSo and 100 µg Ra-α, each solubilised in 50 µl phosphate buffered saline (PBS). As controls 50 µl rabbit serum and 50 µl PBS were injected. The reactions were registered 4, 24 and 48 hr later by measuring the diameter of erythema.

Lymphocyte stimulation- Before killing, the rabbits were bled from the marginal ear vein. The lymphocytes were isolated from the heparinised blood as described earlier (3). Lymphocytes were cultured with 500 µg Ra-LeSo or 50 µg Ra-α. The degree of stimulation was measured in counts per minute (cpm) by the incorporation of ^{3}H-thymidine and expressed as stimulation index (S.I.):

$$\text{S.I.} = \frac{\text{cpm measured in cells cultured in presence of antigen}}{\text{cpm measured in cells cultured in absence of antigen}}$$

Aqueous humor- Immediately after death an 150 µl sample of aqueous humor was withdrawn from both eyes through a 27 gauge needle. The samples were centrifuged and the supernatants used for quantitation of total protein, albumin, sodium, potassium and calcium concentrations. Total protein content was determined by the Lowry method (9) using bovine serum albumin as standard. Sodium, potassium and calcium were determined by standard atomic absorption spectrophotometric techniques. The radial immunodiffusion technique of Mancini et al. (10) was used to determine the quantity of albumin in the aqueous humor. The agarose was mixed with goat anti-rabbit serum albumin antiserum. Rabbit serum was used as standard. The precipitin rings were stained with Coomassie-blue.

RESULTS

The results of both experiments were quite similar. For this reason the results have been presented below as concerning one group of 40 rabbits.

The appearance of the irradiated lenses of the rabbits is demonstrated in Table I. Two days, and 4 and 8 weeks following X-ray treatment no clinical signs of lens opacities were observed as judged by

slit-lamp examination. Twelve weeks after irradiation, 3 rabbits demonstrated clusters of small peripheral subcapsular vacuoles. One rabbit showed subcapsular vacuoles and linear opacities over the whole lens surface. A total opaque lens was present in 7 rabbits killed 20 weeks after irradiation. One rabbit suffered from cortical cataract at that moment.

Table I. *The appearance of rabbit lenses after different periods following X-irradiation.*

Rabbit no.	Period	Observation
1- 8	2 days	clear
9-16	4 wks	clear
17-24	8 wks	clear
25-28	12 wks	clear
29		cortical opacities
30		subcapsular vacuoles
31		subcapsular vacuoles
32		subcapsular vacuoles
33	20 wks	cortical opacities
34-40		mature cataract

Skin tests- The average diameters of erythema measured 24 hr following intradermal injection of Ra-LeSo and Ra-α, after different time intervals following irradiation, are demonstrated in Fig. 1. The most intense skin reaction for both antigen preparations was present, on the average, 8 weeks after irradiation of the rabbits. At this moment no large difference existed in the skin reaction registered 4 or 24 hr after intradermal antigen injection. During the following 4 weeks the skin reaction decreased sharply for Ra-α. The skin reaction registered 48 hr after intradermal injection of Ra-LeSo or Ra-α was always less intense as that registered 24 hr after injection. Control skin tests were always negative.

Lymphocyte stimulation- After different periods following lens irradiation, lymphocytes were isolated and incubated with homologous lens crystallins or alpha crystallin. An optimal stimulation with both

antigen fractions was found 8 weeks following X-ray treatment. The alpha crystallin fraction appeared to be less active (Fig. 2). These results suggest the presence of lymphocytes predominantly sensitised to low molecular weight lens proteins (proteins other than intact alpha crystallin) in the blood of the irradiated rabbits.

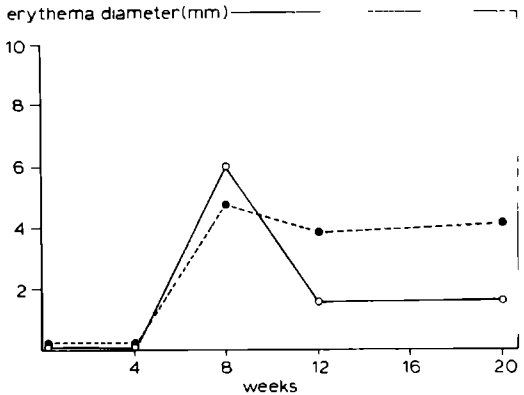

Fig. 1. *Skin reactivity measured 24 hr after intradermal injection of homologous lens proteins (.....) and alpha crystallin (———) in rabbits.*

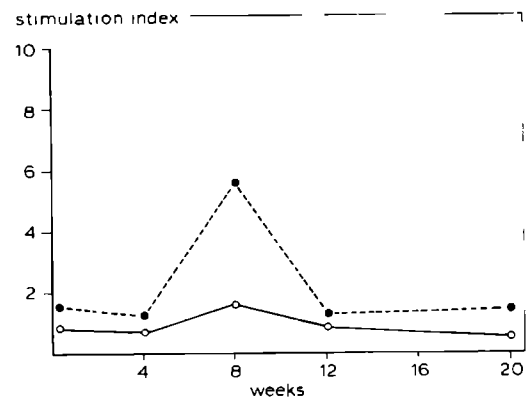

Fig. 2. *Stimulation of rabbit lymphocytes with homologous lens crystallins (.....) and alpha crystallin (———).*

Aqueous humor– The total protein concentration and albumin concentration in the aqueous humor of both eyes was determined simultaneously. The change during the observation period in the mean total protein concentration ratio OD/OS after irradiation is given in Table II. Two days after irradiation the protein concentration ratio was 1.6 and then declined to 1.1 during the following 8 weeks. From then the ratio

increased, reaching an average value of 7.0, 20 weeks after irradiation. The mean albumin concentration ratio OD/OS ran parallel to the total protein concentration ratio OD/OS during the experimental period. Increased values were observed 2 days and from 12 weeks following irradiation of the right eyes (Table II).

Table II. *OD/OS ratios of aqueous humor total protein and albumin concentrations after different periods following X-irradiation of the right eye lenses.*

	Period after irradiation (weeks)				
	0.3	4	8	12	20
Total protein	1.6 ± 0.6	1.1 ± 0.3	1.1 ± 0.3	2.3 ± 1.6	7.0 ± 6.6
Albumin	5.2 ± 1.9	1.0 ± 0.1	1.4 ± 0.5	2.7 ± 1.6	5.1 ± 5.7

Values represent mean OD/OS ratios ± S.D. of 8 rabbits.

The mean OD/OS ratios of the aqueous humor sodium, potassium and calcium concentrations are shown in Table III. During the experimental period no significant differences were observed in sodium or potassium concentration in the aqueous humor (expressed as OD/OS ratio) between the groups of irradiated animals and a group of non-irradiated (control) animals ($P>0.1$). For calcium, however, a statistically significant increase in the OD/OS concentration ratio was found 4 weeks following irradiation of the right lenses as compared to the OD/OS calcium concentration ratio in control animals ($P<0.005$). At the other observation moments no significant differences in aqueous humor calcium concentration (expressed as OD/OS ratio) were found between irradiated and non-irradiated rabbits ($P>0.2$).

DISCUSSION

The goal of the present communication was to investigate the possibility that leakage of lenticular antigens precedes the clinical signs of lens opacity. For this purpose we chose the experimental ra-

Table III. *Ratios (OD/OS) of the aqueous humor sodium, potassium and calcium concentrations between irradiated right eyes (OD) and control left eyes (OS) of rabbits after different periods following X-irradiation.*

Periods after irradiation (weeks)	Sodium	Potassium	Calcium
0.3	1.00 ± 0.23	0.99 ± 0.26	0.82 ± 0.32
4	0.99 ± 0.11	1.03 ± 0.13	1.69 ± 0.65*
8	1.01 ± 0.11	1.16 ± 0.10	1.08 ± 0.47
12	1.10 ± 0.22	1.23 ± 0.18	0.95 ± 0.19
20	0.88 ± 0.25	1.04 ± 0.16	1.03 ± 0.32

Values represent mean OD/OS values ± SD of 8 rabbits.

* Statistically significant different ($P<0.005$) as compared to a non-irradiated group of rabbits (n = 9)
(OD/OS ratios in this group: sodium: 1.06 ± 0.22; potassium: 1.05 ± 0.17; calcium: 0.95 ± 0.11).

diation cataract because lens irradiation resulted in metabolic and structural changes which were also observed during the ageing process (6, 8).

X-irradiation of the lenses in young rabbits resulted in sensitisation of lymphocytes to lens proteins as measured with skin reactions and lymphocyte stimulation. While this sensitisation reached a maximum about 8 weeks following X-ray treatment, clinically, lens opacities could be detected not earlier than 12 weeks following irradiation. These results suggest the leakage of lens proteins out of the lens before the clinical manifestation of the cataract.

Lymphocyte stimulation is a reliable parameter for the detection of cell-mediated immunity in rabbits (3). Moreover, the positive skin reaction 24 hr following intradermal crystallin injection suggests that this type of immune response plays a dominant role in the irradiated rabbits. The fact that we could not detect anti-lens protein antibodies in the serum of the rabbits by hemagglutination tests

(data not shown) supports this.

The difference in lymphocyte stimulation between purified intact alpha crystallin and total lens crystallins suggests that predominantly low molecular weight lens proteins have been leaking out of the lens. These proteins consisted probably of beta or gamma crystallins which are known to be as antigenic as alpha crystallin (12) and were very likely responsible for the autoimmunisation.

The slight increase in OD/OS ratio of aqueous humor protein concentration 2 days following irradiation of the right lenses is probably the result of an inflammatory process of the iris, having a maximal intensity shortly after irradiation (13). The increased amount of albumin in the aqueous humor of the irradiated eyes at this moment also points in this way. According Fukami et al. (5) irradiation of the rabbit eye with 2000 Rad results in damage of blood vessels in iris and ciliary body. This damage exhibits a maximum 7 to 9 weeks following irradiation of the eye. In our experiments, this phenomenon may explain the increase in protein content (including albumin) in the aqueous humor of the right eyes, 12-20 weeks following irradiation with the slightly lower dose of 1800 Rad.

The leakage of low molecular weight lens proteins might be related to the increased concentration of calcium in the aqueous humor encountered 4 weeks following lens irradiation. Duncan and van Heyningen (4) found that during dialysis of lens homogenates against isosmotic buffer, sodium diffused much faster than calcium indicating a relatively free state of sodium and a bound state of calcium in the lens. In accordance with their finding, Jedziniak et al. (7) demonstrated the presence of calcium bound to high and low molecular weight proteins in the lens. In our experiments, the leaking calcium could likewise be bound to protein, restricting it in its permeability through the blood-aqueous barrier and making it detectable as a consequence of its long presence in the aqueous humor (Table III). In contrast, the relatively rapid exchange of free sodium and (probably) potassium ions made detection of alterations of their aqueous humor concentrations impossible in our experiments.

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The human uvea is one of the most vascularised tissues of the eye. Consequently, it is not surprising that an inflammatory process is often manifested in this tissue. From experimental studies performed in several laboratories, it is known that the uvea may behave like a lymph node, especially concerning its capacity to store memory cells. These memory cells are partly responsible for the fast and violent inflammation that sometimes occurs after contact with the appropriate antigens. The origin of these antigens may be extra-ocular as well as intra-ocular.

The lymph node function of the uvea and its high degree of vascularisation partly explain why certain systemic diseases may be accompanied by uveitis of variable intensity. This type of uveitis may be described as secondary uveitis. It has been demonstrated that humoral as well as cellular immune activity can be responsible for this form of uveitis (Chapter 2).

In contrast, the primary form of uveitis, is considered to represent a direct immunological activity to uvea-specific proteins. Viral or bacterial infections or chemical and/or physical influences are possible causes of this type of autoimmune response. These factors may either alter the antigenic properties of the individual protein structures or they trigger the release of proteins which are unknown to the immunocompetent system. The latter may explain the occurrence of a cellular immune response to autologous corneal proteins in patients suffering from keratitis caused by herpes simplex virus infection (Chapter 9).

The tests for cellular immune reactions, described in this thesis, can be helpful to differentiate between the primary and secondary form of uveitis by using antigens of various tissues. This appears to be important particularly with respect to the diagnosis and therapy of the disease, since uveitis occurring as a consequence of either rheumatoid arthritis or serum sickness requires another thera-

peutic approach than uvea destruction caused by uvea-specific protein-sensitised lymphoid cells. By testing proteins isolated from several eye tissues more information about certain immunopathological specificities of the manifestations may be found. Furthermore, one should be aware that symptoms of ocular disorders may sometimes be restricted but nevertheless are the cause of severe complaints of the patients such as decrease of visual acuity or pain. The restricted manifestation of the immune reaction may explain some negative test results.

The in-vitro cellular immunity tests have been recommanded as a suitable method for the detection of immunologic reaction patterns of individual patients. However, results of these immunodiagnostic tests are only reliable when the individual patients are followed up several times within a certain period. Within this period, the patient should neither be submitted to operation nor undergo a change in therapy such as corticosteroid treatment. The importance of surgical interventation has been demonstrated for patients who underwent intracapsular lens extraction and subsequently exhibited a rapid decrease in the cellular immune response to lenticular antigens (Chapter 10). For the correct judgement, it is essential to compare the results of clinical tests with figures obtained with normal control persons under the same technical conditions. Nevertheless, the tests for cellular immunity are proven to be suitable for the study and characterisation of various ocular disorders as shown in patients suffering from retinitis pigmentosa (Chapter 4). While in individual patients the results of the leukocyte migration inhibition test and the lymphocyte stimulation test were not always corresponding, statistically, the results of both tests revealed the probability that this disease is accompanied by an increase in the cellular immune activity to retinal antigens. Moreover, the increased response to rhodopsin resulted in a new hypothesis of the pathogenesis of retinitis pigmentosa.

Another example of the application of these tests is the demonstration of the effects of different types of cataract extraction on the cellular immune status of patients. From these results some criticism raises concerning the corticosteroid treatment after intracapsular lens extraction. This criticism appears also be appropriate for corneal transplantation since, in our clinical department, the

in-vitro assays did not reveal keratoplasty to evoke a significant cellular immune activity to corneal antigens. Thus, from the immunological point of view, it is suggested to abstain from corticosteroid therapy in keratoplasty.

Phacogenic uveitis is a typical example of secondary uveitis. The immune response developing in the uvea is primarily directed to autologous lens proteins released in large quantities during lens trauma or cataract extraction. Studies on the cellular immunity in patients suffering from cataract showed an immune reactivity mainly directed to low molecular weight lens proteins (Chapter 10). Extracapsular lens extraction resulted in an increase in the degree of sensitisation to these lens proteins. The number of patients with positive reactions to lens proteins was increased after this type of cataract extraction as compared to the situation before the operation. In contrast intracapsular lens extraction diminished the percentage of patients showing a positive immune response to lens proteins as compared to the immunologic state before operation. Thus, with respect to the immunologic sequelae, intracapsular lens extraction is preferable to extracapsular lens extraction. Newer techniques, however, make it possible to do such a perfect extracapsular lens extraction that virtually only capsular remnants remain.

Experiments with irradiation cataract revealed the activation of the cellular immune system to be present before the cataract was clinically detectable (Chapter 11). This autoimmune response probably could emerge because of a change in the permeability of the lens capsule resulting in the leakage of low molecular weight lens proteins. It remains uncertain whether in man by a similar increase in permeability an immune response is triggered which in turns influences the progression of the cataract. There exists the possibility of a disturbing influence of permeable products of immune reactions on the lens or of sensitised T-lymphocytes on the lens capsule. Experimental uveitis may be accompanied with lens opacities, a phenomenon which is also encountered in the human eye (complicated posterior subcapsular cataract in uveitis). In addition, the leakage of lenticular proteins could disturb the intra-lenticular environment through inducing the formation of aggregates of remaining lenticular proteins. High molecular weight lens protein aggregates are known to be res-

ponsible for increased light scattering. A more detailed investigation of the role of changes in lens permeability might result in a better understanding of the pathogenesis of cataract.

The inflammatory process after cryotherapy sometimes occurring in patients suffering from retinal detachment is another example of secondary uveitis. It has been demonstrated that patients suffering from retinal detachment for a period of 4 weeks occasionally develop cellular immune activity to retinal proteins (Chapter 3). Renewed contact between memory cells in the uvea and retinal proteins or protein fragments released during cryotherapy might initiate the course of inflammatory events as it is sometimes seen in patients following this therapy.

Viral infection, resulting in corneal cell damage, may explain the increased cellular immune response to corneal proteins of patients suffering from herpes simplex virus keratitis. The development of an autoimmune reaction to proteins from the affected tissues in patients suffering from bullous keratopathy, cataract, retinal detachment or retinitis pigmentosa is more difficult to understand. Numerous investigators suggest the lens, cornea, vitreous body and retinal pigment epithelium to be in an immunologically privileged state because they lack both blood supply and lymphatic drainage, thus being unrecognizable for immunocompetent cells (hidden antigen theory). Only upon leakage of their specific antigenic determinants these tissues are considered to display antigenicity. However, antigenic determinants of proteins of corneal epithelium (Chapter 8), lens (alpha crystallin) and retina (rhodopsin) (Chapte 6) are present in several vascularised eye tissues as well. Therefore, the location of these proteins by which they are shielded from immunocompetent cells as the sole mechanism in protection from autosensitisation appears to be questionable. The phenomenon of immunologic tolerance seems to play a more important role in the onset of several of the observed cellular immune responses. Assuming the existence of a threshold of immunologic tolerance for each autologous protein, the exceeding of the threshold for retinal proteins during retinal detachment or retinitis pigmentosa could result in an autoimmune response. This may also be valid for lens proteins leaking out of the lens during cataract progression or for corneal proteins leaking

out of the oedematous cornea in bullous keratopathy.

The presence of a common antigenicity among various eye tissues explains the complexity of some ocular disorders. Moreover, common antigenic determinants of intra- and extra-ocular tissues are likely to result in the involvement of the particular eye tissues in a systemic disorder such as rheumatoid arthritis. Cellular immune sensitivity to type II collagen has been demonstrated in patients suffering from rheumatoid arthritis. This type of collagen is present too, in cornea, vitreous body and retina which may explain the common ocular involvement in this disease.

Only a few ocular diseases are considered to have a real underlying autoimmune mechanism. Sympathetic ophthalmia and phacoanaphylactic uveitis are two of them. The diseases studied in this thesis, i.e. retinal detachment, retinitis pigmentosa, keratitis and senile cataract are probably secondarily associated with autoimmune activity. Nevertheless, knowledge about the involvement of the immune system in these diseases may bring more insight in the pathogenesis of these disorders and proposals for their therapy. For this purpose in-vitro studies have been performed using isolated lymphocytes or leukocytes taking in consideration that such an isolated system does not display the complexity of the immune response in-vivo. The majority of immune reactions are the result of the activity of the cellular immune system as well as the humoral immune system with possible predomination of one type.

The clinical investigations described in this thesis are considered to be orientating studies to screen immunologically different types of ocular disorders. From the results other problems have risen of which the clarification appears to be essential for further understanding of ocular (immuno-)pathology. Some of the problems to be clarified are the identification of the involved antigens in retinal detachment and keratitis, the question about the primary or secondary involvement of the cellular immune system in retinitis pigmentosa, and the existence of any correlation between the type of retinal degeneration and the cell-mediated immune response. Animal experiments associated with clinical laboratory studies are proposed to answer these questions, since one of the greatest problems encountered in ophthalmic research is the limited availability of hu-

man eye tissues for test-antigen preparation which makes the use of animal material inevitable. The chief goal of further studies should be to achieve information about animal test antigens which can replace the respective human antigens and to ascertain the extent of correlation between the experimental model and the human disease.

This thesis deals with investigations on the involvement of the immune system in various human ocular disorders. Animal experiments have been performed in order to get more insight into the pathogenesis of clinical phenomena. The major part of the work was done with in-vitro tests in able to detect cell-mediated immune reactions in human beings and in rabbits. The lymphocyte stimulation test and the leukocyte migration inhibition test were made suitable for our purposes.

Chapter 1- The conditions are described required for optimal lymphocyte stimulation and leukocyte migration inhibition. Large differences appeared to be present between human and rabbit lymphocytes with respect to culture times and antigen concentrations for different types of antigens.

Chapter 2- Chapter 2 deals with the relative importance of the humoral- and the cell-mediated immune system in endogenous uveitis. Transfer of immune serum or sensitised thymocytes to unsensitised rabbits, followed by intravitreal injection of the corresponding antigen, resulted both in clinically detectable inflammatory processes. Histologically, by the transfer of immune serum phenomena were evoked resembling an Arthus-type reaction. The uveal infiltration consisted predominantly of polymorphonuclear leukocytes. The transfer of sensitised homologous thymocytes resulted in an uveal inflammation with characteristics of the delayed-type hypersensitivity reaction. Tissue infiltration of polymorphonuclear as well as mononuclear cells was observed.

Chapter 3- Several investigators succeeded in inducing experimental uveitis by injecting retinal extracts in animals, indicating the antigenic nature of this tissue. Release of retinal antigens during retinal detachment might similarly induce an immune reaction in patients. A positive lymphocyte stimulation with retinal antigens could be detected in 41% of all patients suffering from retinal de-

tachment. The occurence of sensitised lymphocytes depended on the duration of the detachment. They could be demonstrated in 57% of patients suffering from this disease for more than four weeks. No correlation between the presence of sensitised lymphocytes and a positive antibody titer to retinal antigens was found. Lymphocytes of controls were inactive upon incubation with retinal antigens. Anti-retinal antigen antibodies were not detectable in the controls.

Chapter 4 - Attempts have been undertaken to determine more precisely the immunogenicity of different retinal fractions. Soluble human retinal antigens and bovine rod outer segments appeared to possess stimulatory capacity upon incubation with lymphocytes of patients suffering from retinitis pigmentosa. Human insoluble retinal antigens, devoided of photorecepter fragments, an uveal pigment granules were inactive in this test. Leukocytes of patients with retinitis pigmentosa reacted positively with purified rhodopsin. On the basis of these findings it was concluded that patients suffering from retinitis pigmentosa may become sensitised to retinal antigens especially to those localised in the rod outer segments. This sensitisation was related to the cell-mediated immune reaction and seemed not to be correlated with a special type of the disease.

Chapter 5 - Since one of the most intricated problems in ophthalmic research is the limited availability of suitable human eye tissues for antigen preparation, we have to recourse to animal tissues. Therefore, in Chapter 5, results of studies on the common antigenicity between the retinas of several animal species, as revealed by different immunological techniques, are described. Lymphocytes of rabbits sensitised to human soluble retinal antigens were equally stimulated by human, monkey and pig retinal extracts whereas bovine retinal antigens evoked a significantly lower stimulation. Lymphocytes of patients, suffering from retinitis pigmentosa reacted in an identical way with human and pig retinal extracts. Immunodiffusion, immunoelectrophoresis and immunoelectrofocusing revealed the presence of common antigenic determinants in soluble retinal fractions of man and monkey (Macaca fascicularis). Basic retinal proteins of the tested species formed no precipitin lines with rabbit anti-human retina antiserum. From this study it was concluded that the soluble retinal extracts of monkey and man showed the closest common anti-

genicity.

Chapter 6 - The stimulatory potentiality of rhodopsin on the cellular immune system has been shown by injecting rabbits with this lipoglycoprotein. In contrast, bovine alpha crystallin predominantly activated the humoral immune system in these animals. Furthermore by incubating rhodopsin - or alpha crystallin-sensitised rabbit lymphocytes with extracts of various ocular tissues, it was shown that rhodopsin determinants appeared to be present in cornea, sclera and the soluble part of the retina. Alpha crystallin determinants were detectable in iris, sclera and choroid.

Chapter 7-8 - Similar studies have been performed with lymphocytes sensitised to bovine corneal epithelium. Determinants of soluble corneal epithelium-proteins were found in the insoluble membraneous part of the corneal epithelium (Chapter 7) and in the soluble fractions of corneal stroma,lens epithelium, iris, retina and sclera (Chapter 8).

Chapter 9 - The presence of lymphocytes sensitised to rhodopsin in patients suffering from retinitis pigmentosa has been described above. Thus, the common antigenicity of rhodopsin and some other ocular tissues might explain the complexity often encountered in this retinal disease. This may also be valid for patients suffering from corneal disease. In instances of corneal disorders, the lymphocytes of some patients reacted with homologous corneal antigens, especially the lymphocytes of patients suffering from herpes simplex virus keratitis. Antigens isolated from bovine corneal epithelium displayed more stimulatory activity than those isolated from bovine corneal stroma. This activity concerned both the histocompatibility- as well as the non-histocompatibility antigens. Therefore, it was suggested that elimination of donor corneal epithelium before transplantation may be beneficial.

Chapter 10 - Senile cataract may also be accompanied by an increased state of immune activity. Lymphocytes of patients suffering from cataract reacted in 38% of all cases with total human lens crystallins and in 15% of all cases with purified bovine alpha crystallin. This difference in sensitisation suggested leakage of lenticular antigens other than alpha crystallin out of the lens during

cataract progression. Extra-capsular lens extraction seems to increase the risk of sensitisation since the number of patients with lymphocytes positively reacting to total lens crystallins and alpha crystallin was higher after this operation as compared to untreated patients. Less incidences of sensitisation to lens crystallins secondarily to intra-capsular lens extraction as compared to untreated patients were observed.

Chapter 11 - At present it is hardly to answer whether leakage of human lenticular antigens precedes the clinical signs of lens opacities or vice versa. The alterations in radiation cataract show some similarities to those in human senile cataract. Irradiation of the lens of one eye in young rabbits resulted in sensitisation of lymphocytes to lens proteins probably of low molecular weight. While this sensitisation reached a maximum about 8 weeks following X-ray treatment, clinically, lens opacities could be detected not earlier than 12 weeks following irradiation. These results suggested the leakage of low molecular weight lens proteins or protein fragments out of the lens before the clinical manifestation of the cataract.

Dit proefschrift beschrijft het onderzoek naar de betrokkenheid van het immuun systeem bij verschillende oogaandoeningen. Ten einde meer duidelijkheid te verkrijgen omtrent de klinische resultaten werd eveneens dierexperimenteel onderzoek verricht. Het grootste deel van het onderzoek naar de aanwezigheid van een cellulaire immuun reactie bij mensen en konijnen werd uitgevoerd door gebruik te maken van in-vitro testen. Deze testen, de lymfocyten stimulatie test en de leukocyten migratie inhibitie test, werden voor dit doel geschikt gemaakt.

Hoofdstuk 1 - De omstandigheden, welke noodzakelijk zijn voor optimale lymfocyten stimulatie en leukocyten migratie inhibitie worden beschreven. Er bleek een groot verschil te bestaan in kweektijd en optimale antigeen concentratie tussen menselijke en konijnelymfocyten, terwijl de verschillende antigene fracties zeer uiteenlopende stimulerende activiteiten ten opzichte van deze lymfocyten vertoonden.

Hoofdstuk 2 - Hoofdstuk 2 beschrijft de relatieve invloed van de humorale en cellulaire immuniteit op het ontstaan van experimentele endogene uveitis. Passieve immunisatie door overdracht van antiserum, respectievelijk gesensibiliseerde thymocyten naar konijnen, gevolgd door een intravitreale injectie met het corresponderende antigeen resulteerden in beide gevallen in een klinisch waarneembaar ontstekingsproces. Histologisch onderzoek van de ogen toonde een typische Arthus reactie aan in de konijnen, welke antiserum gekregen hadden. Overdracht van gesensibiliseerde thymocyten daarentegen resulteerde in een uveitis, welke kenmerken van een vertraagde overgevoeligheidsreactie vertoonde. Hierbij namen we infiltraties van zowel polymorfonucleaire als mononucleaire cellen waar.

Hoofdstuk 3 - In verschillende laboratoria is men er in geslaagd bij proefdieren uveitis te induceren door middel van injec-

tie met retina extracten, hetgeen een ondersteuning is van de vermoede antigeniciteit van de retina. Retina antigenen, welke gedurende retina loslating vrijkomen, zouden een immuun reactie kunnen veroorzaken bij patienten. Wij vonden een positieve lymfocyten respons bij 41% van de geteste patienten, welke aan retina loslating leden. De mate van sensibilisatie hing af van de duur van de loslating en kon worden aangetoond in 57% van alle patienten, welke langer dan 1 maand een retina loslating hadden. Tussen de aanwezigheid van gesensibiliseerde lymfocyten en een positieve antilichamen titer tegen retina antigen bleek geen correlatie te bestaan. De lymfocyten van controle personen reageerden niet met retina antigen. Anti-retina eiwit antilichamen konden evenmin bij deze personen worden aangetroffen.

Hoofdstuk 4 - Wij hebben tevens getracht de aard van verschillende retina fracties nader te bepalen. De oplosbare antigenen van menselijke retina's en de staafjes-buitensegmenten van runder retinas bleken stimulerend te werken op lymfocyten van patienten, welke leden aan retinitis pigmentosa. De onoplosbare fractie van menselijke retina's (welke geen fotoreceptor fragmenten bevatten) en runder uvea pigmentkorrels reageerden daarentegen negatief in deze test. De leukocyten van patienten met retinitis pigmentosa reageerden positief met gezuiverd rhodopsine. Deze resultaten deden vermoeden, dat patienten welke aan retinitis pigmentosa lijden, gesensibiliseerd kunnen raken tegen retina antigenen, welke voornamelijk gelocaliseerd zijn in de fotoreceptorlaag. Deze sensibilisatie betrof vooral het cellulaire immuun systeem en was niet gecorreleerd met een speciaal type van de ziekte. De uitgebreidheid van de aandoening gemeten door middel van het electro-retinogram was echter wel van invloed.

Hoofdstuk 5 - Een van de grootste problemen waarmee men in de oogheelkundige research te kampen heeft is het tekort aan menselijke oogweefsels voor de bereiding van antigenen. Dit maakt het gebruik van dierlijk weefsel noodzakelijk. In hoofdstuk 5 wordt de gemeenschappelijke antigeniciteit beschreven tussen de retina's van verschillende diersoorten als aangetoond met verschillende immunologische technieken. Lymfocyten van konijnen, welke gesensibiliseerd

waren voor humane oplosbare retina antigenen bleken in dezelfde mate gestimuleerd te kunnen worden door de retina extracten van mens, aap en varken. Runder retina extract gaf een significant lagere stimulering. Lymfocyten van patienten met retinitis pigmentosa reageerden in gelijke mate met humaan en varkens retina extract. Door middel van immunodiffusie, immunoelectroforese en immunoelectrofocusing werd de aanwezigheid van gemeenschappelijke antigene determinanten in de oplosbare fractie van mens en aap (Macaca fascicularis) aangetoond. Geen van de basische eiwitten van de geteste diersoorten vormden een precipitatie lijn met konijn-anti-human retina-antiserum. Wij concludeerden uit dit onderzoek, dat de oplosbare retina extracten van mens en aap de nauwste gemeenschappelijke antgeniciteit bezitten.

Hoofdstuk 6 - Dat rhodopsine vooral het cellulaire immuun systeem stimuleert, kon worden aangetoond door konijnen met dit lipoglycoprotein in te spuiten. Runder alfa crystalline daarentegen bleek vooral het humorale systeem van deze dieren te activeren. Door konijnelymfocyten, gesensibiliseerd voor rhodopsine, met verscheidene oogweefsel extracten te incuberen kon worden aangetoond, dat rhodopsine-determinanten aanwezig zijn in de cornea, de sclera en de oplosbare fractie van de retina. Op soortgelijke manier werd aangetoond dat alfa crystalline-antigene determinanten aanwezig zijn in de iris, sclera en choroidea.

Hoofdstuk 7-8 - Dergelijke studies zijn tevens uitgevoerd met lymfocyten, welke gesensibiliseerd waren voor de oplosbare eiwitten uit runder cornea epitheel. Deze eiwitten of bepaalde antigene determinanten hiervan bleken aanwezig in de onoplosbare fractie van het cornea epitheel (hoofdstuk 7) en in de oplosbare fractie van cornea stroma, lens epitheel, iris, retina en sclera (hoofdstuk 8).

Hoofdstuk 9 - De aanwezigheid van lymfocyten gesensibiliseerd voor rhodopsine in patienten met retinitis pigmentosa is reeds beschreven. De waargenomen gemeenschappelijke antigeniciteit tussen rhodopsine en sommige andere oogweefsels kan een verklaring zijn voor ontstekingen van gecompliceerd karakter, welke vaak bij retinitis pigmentosa wordt aangetroffen. Deze verklaring van complicaties zou ook kunnen gelden voor bepaalde cornea ziekten. Lymfocyten

van enkele patienten met cornea aandoeningen reageerden op homologe cornea antigenen. Dit was vooral het geval met lymfocyten van patienten, welke aan herpes simplex virus keratitis leden. Antigenen, welke uit het cornea epitheel geisoleerd waren, bleken een sterker stimulerend vermogen te bezitten dan antigenen uit het cornea stroma. Dit stimulerend vermogen betrof zowel de histocompatibiliteits antigenen als de niet-histocompatibiliteits antigenen. Dit betekent dat verwijdering van het donor cornea epitheel voor de transplantatie de kans op succes zou kunnen vergroten.

Hoofdstuk 10 - Seniele cataract kan eveneens vergezeld gaan van een verhoogde staat van immuun activiteit. De lymfocyten van patienten, welke leden aan dit type van cataract, reageerden in 38% van alle gevallen met de totale lenscrystalline fractie en in 15% met gezuiverd alfa crystalline. Dit verschil in gevoeligheid ten opzichte van beide crystalline fracties suggereerde een lekkage van andere eiwitten dan alfa crystalline uit de lens tijdens cataract progressie. Extracapsulaire lens extractie resulteerde in een toename van het aantal patienten dat reageerde met de totale lenscrystalline fractie en met alfa crystalline. Intracapsulaire lens extractie daarentegen resulteerde in een verminderde gevoeligheid voor lens crystallines vergeleken met nog niet geopereerde patienten.

Hoofdstuk 11 - Een antwoord op de vraag of lekkage van eiwitten door het kapsel van de menselijke lens voorafgaat aan de klinische tekenen van lenstroebeling is moeilijk te geven. Om dit te kunnen bestuderen kozen wij de stralingscataract als model, omdat de veranderingen enige gelijkenis vertonen met die in de humane seniele cataract. Bestraling van de rechter ooglens van jonge konijnen resulteerde in een gevoeligheid van lymfocyten voor laag-moleculaire lens eiwitten. Deze gevoeligheid bleek maximaal aantoonbaar te zijn, 8 weken na de röntgen bestraling. Lenstroebelingen konden klinisch niet eerder worden waargenomen dan 12 weken na de bestraling. Deze resultaten deden vermoeden, dat er reeds lekkage optreedt van laag-moleculaire lens eiwitten of van eiwitfragmenten voordat cataract klinisch waarneembaar is.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 12 maart 1950 te Den Helder. Hij bezocht de HBS-B te Waalwijk en Amsterdam alwaar hij het einddiploma behaalde in 1969. In hetzelfde jaar ving hij aan met de scheikunde studie aan de Universiteit van Amsterdam en legde het kandidaatsexamen af in 1972. Zijn doctoraal studie (Biochemie) voltooide hij in 1975 aan de Rijks Universiteit van Groningen. Aan deze universiteit legde hij tevens, in 1974, het kandidaatsexamen farmacie af. Vanaf 1 januari 1976 was hij verbonden aan de kliniek voor Oogheelkunde van het St. Radboud Ziekenhuis te Nijmegen waar de werkzaamheden, beschreven in dit proefschrift, werden uitgevoerd. In 1978 is een aanvang genomen met de studie Geneeskunde aan de Katholieke Universiteit van Nijmegen. Sinds 1 januari 1980 is hij werkzaam op de afdeling Neurologie van het St. Radboud Ziekenhuis om onderzoek te verrichten naar de immunopathologie van multipele sclerose.

STELLINGEN

1. Het is onjuist te beweren, dat lens eiwitten in extra-lenticulaire weefsels voorkomen op grond van immunologische reacties tussen deze weefsels en anti-lens eiwit antiserum.

2. De conclusie, dat calcium geen invloed heeft op de regeneratie van rhodopsine uit opsine en 11-cis-retinal in onjuist doordat dit regeneratie proces slechts bestudeerd werd bij 4°C.
Nöll et al. Biophys. Struct. Mechanism 5, 33, 1979.

3. De mogelijkheid, dat retinitis pigmentosa zijn oorsprong vindt in een genetische afwijking, welke tot een mutatie in de primaire struktuur van rhodopsine leidt, dient overwogen te worden.

4. Het lijkt onjuist te veronderstellen, dat het succesvolle gebruik van gepreserveerde middenoor implantaten te danken is aan een vermindering of een totale afwezigheid van weefselantigenen in het gepreserveerde weefsel.
Perkins, Tr.Am.Acad.Ophthal. & Otol. 85, 337, 1975.

5. Het is niet uitgesloten, dat het oplossen van lensmembraan lipiden in 7 M ureum zoals beschreven door Cotlier et al. dient te worden toegeschreven aan het achterblijven van een membraanfractie na centrifugering van de 7 M ureum suspensie van de membranen.
Cotlier et al., B.B.A. 530, 267, 1978.

6. De remmende aktiviteit van liquor cerebrospinalis van multipele sclerose patienten op de migratie van macrofagen of getanneerde schape-erythrocyten in een electrisch veld is geen direct bewijs voor de aanwezigheid van lymfokinen in de liquor van deze patienten.
Meyer-Rienecker et al., J. Neurol. Sci. 42, 173, 1979.

7. Het als routine voorschrijven van corticosteroiden na hoornvliestransplantaties ten einde een afstotingsproces te voorkomen is discutabel.

8. Een tentamen statistiek dient in elke studierichting opgenomen te worden.

9. Ten einde de (voetbal)sport voor het publiek aantrekkelijker te maken zou men het aantal gemaakte doelpunten in de competitiestand dienen te verwerken.

C.J.J. Brinkman

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